EXHIBIT A

1. A unit dose composition for inducing angiogenesis in a human, comprising about .008 mg to about 7.2 mg of FGF-2 or an angiogenically active fragment or mutein thereof in a pharmaceutically acceptable carrier.

2 The unit dose composition of claim 1, comprising 0.3 mg to 3.5 mg of FGF-2, or an angiogenically active fragment or mutein thereof.

3. The unit dose composition of claim 1, wherein said FGF-2 has the amino acid sequence of SEQ ID NO: 2.

4. The unit dose composition of claim 3, comprising 0.3 mg to 3.5 mg of an FGF-2 of SEQ ID NO: 2 or an angiogenically active fragment or mutein thereof in a pharmaceutically acceptable carrier.

5. The unit dose composition of claim 3, comprising about .008 mg to about 7.2 mg of said angiogenically active mutein of said FGF-2 of SEQ ID NO: 2 in a pharmaceutically acceptable carrier.

6. The unit dose composition of claim 5, comprising 0.3 mg to 3.5 mg of said angiogenically active mutein of said FGF-2 of SEQ ID NO: 2 in a pharmaceutically acceptable carrier.

7. The unit dose composition of claim 3, comprising about .008 mg to about 7.2 mg of said angiogenically active fragment of said FGF-2 of SEQ ID NO: 2 in a pharmaceutically acceptable carrier.

8. The unit dose composition of claim 7, comprising 0.3 mg to 3.5 mg of said angiogenically active fragment of said FGF-2 of SEQ ID NO: 2 in a pharmaceutically acceptable carrier.

9. The unit dose composition of claim 3, comprising about .008 mg to about 7.2 mg of FGF-2 of SEQ ID NO: 2 in a pharmaceutically acceptable carrier in a pharmaceutically acceptable carrier.

10. A method for treating a human patient for coronary artery disease comprising, administering a therapeutically effective amount of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof to one or more coronary vessels or to a peripheral vein in a human patient in need of treatment for said coronary artery disease, said therapeutically effective amount being about 0.2 μg/kg to 48 μg/kg of patient weight.

11. The method of claim 10, wherein said recombinant FGF-2 has the amino acid sequence of SEQ ID NO: 2.

12. The method of claim 11, further comprising the step of administering to said human patient about 10 U/kg to 80 U/kg of heparin within about 0 to 30 minutes prior to administering said recombinant FGF-2 of SEQ ID NO: 2 or said angiogenically active fragment or mutein thereof.

13. The method of claim 12, wherein said therapeutically effective amount of a recombinant FGF-2 of SEQ ID NO: 2 or an angiogenically active fragment or mutein thereof is administered to one or more coronary vessels.

14. The method of claim 13, wherein said therapeutically effective amount of a recombinant FGF-2 of SEQ ID NO: 2 or an angiogenically active fragment or mutein thereof is about 24 μg/kg to 48 μg/kg.

15. The method of claim 12 wherein said therapeutically effective amount of a recombinant FGF-2 of SEQ ID NO: 2 or said angiogenically active fragment or mutein thereof is administered to a peripheral vein.

16. The method of claim 15, wherein said therapeutically effective amount of a recombinant FGF-2 of SEQ ID NO: 2 or said angiogenically active fragment or mutein thereof is about 18 μg/kg to 36 μg/kg.

17. A method for treating a human patient for coronary artery disease comprising, administering a single unit dose of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof to one or more coronary vessels or to a peripheral vein in a human patient in need of treatment for coronary artery disease, said unit dose comprising from about .008 mg to 7.2 mg of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof.

18. The method of claim 17, wherein said FGF-2 has the amino acid sequence of SEQ ID NO: 2.

19. The method of claim 18, wherein said single unit dose produces a therapeutic benefit against coronary artery disease in said human patient that lasts at least four months.

20. The method of claim 19, wherein said therapeutic benefit in said human patient lasts 6 months.

21. The method of claim 20, wherein said therapeutic benefit is of such magnitude and duration in said human patient such that administration of a second unit dose is not required for about 6 months.

22. The method of claim 20, wherein said unit dose is administered to one or more coronary arteries.

23. The method of claim 20, wherein said unit dose is administered to a peripheral vein.

24. The method of claim 20, wherein said unit dose comprises 0.3 mg to 3.5 mg of a recombinant FGF-2 of SEQ ID NO: 2 or an angiogenically active fragment or mutein thereof.

25. The method of claim 19, further comprising the step of administering 10 U/kg to 80 U/kg of heparin to said patient IV or IC about 0 to 30 minutes prior to administering said unit dose.

26. A method for inducing angiogenesis in a heart of a human patient comprising, administering a single unit dose of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof to one or more coronary vessels or to a peripheral vein in a human patient in need of treatment for coronary artery disease, said unit dose comprising from about .008 mg to 7.2 mg of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof.

27. The method of claim 26, wherein said FGF-2 has the amino acid sequence of SEQ ID NO: 2.

28. The method of claim 27 wherein said single unit dose produces an improvement in one or more clinical endpoints in said human patient that lasts at least four months.

29. The method of claim 28, wherein said single unit dose produces an improvement in one or more clinical endpoints in said human patient that lasts 6 months.

30. A method for treating a human patient for a myocardial infarction comprising, administering a single unit dose of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof to one or more coronary vessels or to a peripheral vein in said human patient, said unit dose comprising from about .008 mg to 7.2 mg of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof.

31. The method of claim 30, further comprising the step of administering 10 U/kg to 80 U/kg of heparin to said patient IV or IC about 0 to 30 minutes prior to administering said unit dose.

32. The method of claim 31, wherein FGF-2 has the amino acid sequence of the SEQ ID NO: 2.

33. The method of claim 30, wherein said unit dose is administered to a peripheral vein.

34. The method of claim 30, wherein said unit dose is administered into one or more coronary vessels of said patient.

35. A method for providing a human patient with relief from symptoms of angina comprising administering a single unit dose of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof to one or more coronary vessels or to a peripheral vein in a human patient in need of relief from symptoms of angina, said unit dose comprising from about .008 mg to 7.2 mg of a recombinant FGF-2 or an angiogenically active fragment or mutein thereof.

36. The method of claim 35, wherein said unit dose is administered to one or more coronary vessels.

37. The method of claim 35, wherein said unit dose is administered to a peripheral vein.

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optic axis in a crystal not having the same properties in all directions with regard to light, a direction along which there is no apparent double refraction since both components of the light ray have the same velocity
optic disk *same as* BLIND SPOT (sense 1)
op·ti·cian (äp tish'ən) *n.* [Fr. *opticien*] a person who makes or deals in optical instruments, esp. one who prepares and dispenses eyeglasses
optic nerve either of the second pair of cranial nerves, which connect the retina of the eye with the brain
op·tics (äp'tiks) *n.pl.* [*with sing. v.*] [< OPTIC] the branch of physics dealing with the nature and properties of light and vision
op·ti·mal (äp'tə məl) *adj.* [OPTIM(UM) + -AL] most favorable or desirable; best; optimum —**op'ti·mal·ly** *adv.*
op·ti·mism (-miz'm) *n.* [Fr. *optimisme* < L. *optimus*, best (see OPTIMUM)] 1. *Philos. a*) the doctrine held by Leibniz and others that the existing world is the best possible *b*) the doctrine or belief that good ultimately prevails over evil 2. the tendency to take the most hopeful or cheerful view of matters or to expect the best outcome; practice of looking on the bright side of things —**op'ti·mist** (-mist) *n.* —**op'ti·mis'tic** (-mis'tik), **op'ti·mis'ti·cal** *adj.* —**op'ti·mis'ti·cal·ly** *adv.*
op·ti·mize (-miz') *vi.* **-mized'**, **-miz'ing** to be given to optimism —*vt.* to make the most of; develop or realize to the utmost extent; obtain the most efficient or optimum use of —**op'ti·mi·za'tion** *n.*
op·ti·mum (-məm) *n., pl.* **-mums, -ma** (-mə) [L., neut. of *optimus*, best < *ops*, power, riches: for base see OPUS] 1. the best or most favorable degree, condition, amount, etc. 2. *Biol.* the amount of heat, light, moisture, food, etc. most favorable for growth and reproduction —*adj.* most favorable or desirable; best; optimal
op·tion (äp'shən) *n.* [Fr. < L. *optio* < *optare*, to wish, desire, ult. < IE. base **op-*, to choose, prefer] 1. the act of choosing: choice 2. the power, right, or liberty of choosing 3. something that is or can be chosen; choice 4. the right, acquired for a consideration, to buy, sell, or lease something at a fixed price, sign or renew a contract, etc. within a specified time —☆*vt. Sports* to transfer (a player) to a minor league with the option of recalling him —*SYN.* see CHOICE
op·tion·al (-'l) *adj.* left to one's option, or choice; not compulsory; elective —**op'tion·al·ly** *adv.*
op·to·e·lec·tron·ics (äp'tō i lek'trän'iks) *n.pl.* a branch of electronics involving the use of optical technology —**op'to·e·lec'tron'ic** *adj.*
op·tom·e·ter (äp täm'ə tər) *n.* [see OPTIC & -METER] an instrument for determining error in the refractive power of the eye
☆**op·tom·e·trist** (-trist) *n.* a specialist in optometry
op·tom·e·try (-trē) *n.* [see OPTIC & -METRY] 1. measurement of the range and power of vision 2. the profession of examining the eyes and measuring errors in refraction and of prescribing glasses to correct these defects —**op·to·met·ric** (äp'tə met'rik), **op'to·met'ri·cal** *adj.*
op·u·lent (äp'yə lənt) *adj.* [L. *opulentus* or *opulens* < *ops*: see OPUS] 1. very wealthy or rich 2. characterized by abundance or profusion; luxuriant —*SYN.* see RICH —**op'u·lence, op'u·len·cy** *n.* —**op'u·lent·ly** *adv.*
o·pun·ti·a (ō pun'shē ə, -shə) *n.* [ModL. < L. (*herba*) *Opuntia*, (plant) of Opus, city in Locris, Greece] any of a large genus (*Opuntia*) of cactus plants with red, purple, or yellow flowers, pulpy or dry berries, and fleshy, jointed stems, including the prickly pears and chollas
o·pus (ō'pəs) *n., pl.* **o·pe·ra** (ō'pə rə, äp'ər ə), **o'pus·es** [L., a work < IE. **ops* < base **op-*, to work, riches, whence L. *ops*, riches, Sans. *ápas-*, work, OE. *efnan*, to work, do] a work; composition; esp., any of the musical works of a composer numbered in order of composition or publication
o·pus·cule (ō pus'kyōōl) *n.* [Fr. < L. *opusculum*, dim. of *opus*: see prec.] a minor work —**o·pus'cu·lar** *adj.*
·o·py (ō'pē) *same as* -OPIA
☆**o·quas·sa** (ō kwas'ə) *n.* [< *Oquassa* Lake, in Maine] a small trout (*Salvelinus oquassa*) of lakes of W Maine
or[1] (ôr; *unstressed* ər) *conj.* [ME., in form a contr. of *other*, *auther*, either, but actually < OE. *oththe* (in *āther . . . oththe*, either . . . or)] a coordinating conjunction introducing an alternative; specif., *a*) introducing the second of two possibilities [beer *or* wine] *b*) introducing any of the possibilities in a series, but usually used only before the last [apples, (*or*) pears, *or* plums] *c*) introducing a synonymous word or phrase [botany, *or* the science of plants] *d*) introducing the second of two possibilities when the first is introduced by *either* or *whether* [either go *or* stay, whether to go *or* stay] *e*) substituted for *either* as the first correlative ["*or* in the heart *or* in the head"]
or[2] (ôr) *conj., prep.* [ME. < OE. *ār*, var. of *ær*, *ere*: cf. ERE] [Archaic or Dial.] before; ere
or[3] (ôr) *n.* [Fr. < L. *aurum*, gold: for IE. base see EAST] *Heraldry* gold or yellow, represented in engraving by small dots powdered over a plain field
-or (ər; *occas.* ôr) 1. [ME. *-our* < OFr. *-our*, *-or*, *-eur* < L. *-or*, *-ator*] *a n.-forming suffix meaning* a person or thing that [*inventor, objector*] 2. [ME. *-our* < OFr. < L. *-or*] *a n.-forming suffix meaning* quality or condition [*horror, error*]: in Brit. usage, often **-our**
‡**o·ra** (ôr'ə) *n. pl. of* OS[2]
or·ach, or·ache (ôr'əch, är'-) *n.* [ME. *orage* < Anglo-Fr. *orache* < OFr. *arroche* < VL. **atrapica* (for L. *atriplex*) < Gr. *atraphaxys*] any of a genus (*Atriplex*) of plants of the goosefoot family, widespread in salty or alkaline areas, having usually silvery foliage and small green flowers; esp., **garden orach** (*Atriplex hortensis*), cultivated as a potherb, chiefly in France
or·a·cle (ôr'ə k'l, är'-) *n.* [ME. < OFr. < L. *oraculum*, divine announcement, oracle < *orare*, to speak, pray, beseech < *os* (gen. *oris*), the mouth: see ORAL] 1. among the ancient Greeks and Romans, *a*) the place where, or medium by which, deities were consulted *b*) the revelation or response of a medium or priest 2. *a*) any person or agency believed to be in communication with a deity *b*) any person of great knowledge or wisdom *c*) opinion or statements of any such oracle 3. the holy of holies of the ancient Jewish Temple: I Kings 6:16, 19–23
o·rac·u·lar (ō rak'yoo lər) *adj.* 1. of, or having the nature of, an oracle 2. like an oracle; wise, prophetic, mysterious, etc. —**o·rac'u·lar'i·ty** (-yə lar'ə tē) *n.* —**o·rac'u·lar·ly** *adv.*
o·rad (ôr'ad) *adv.* [< L. *os* (gen. *oris*), the mouth + -AD[2]] toward the mouth or oral region
O·ra·dea (ô räd'yä) city in NW Romania, near the Hungarian border: pop. 112,000
o·ral (ôr'əl) *adj.* [< L. *os* (gen. *oris*), the mouth < IE. base **ōus-*, mouth, edge, whence Sans. *á-h*, mouth, ON. *ōss*, mouth of a stream] 1. uttered by the mouth; spoken 2. of speech; using speech 3. of, at, or near the mouth 4. *Phonet.* having mouth resonance only: distinguished from NASAL 5. *Psychoanalysis a*) designating or of the earliest stage of psychosexual development in which interest centers around sucking, feeding, and biting *b*) designating or of such traits in the adult as friendliness, generosity, and optimism or aggressiveness and pessimism, regarded as unconscious psychic residues of that stage: cf. ANAL, GENITAL 6. *Zool.* on or of the same side as the mouth —☆*n.* an examination that is oral and not written, as in a college —**o'ral·ly** *adv.*
***SYN.*—oral** refers to that which is spoken, as distinguished from that which is written or otherwise communicated [an *oral* promise, request, etc.]; **verbal**, though sometimes synonymous with **oral**, in strict discrimination refers to anything using words, either written or oral, to communicate an idea or feeling [a *verbal* image, caricature, etc.]
oral history 1. historical data consisting of personal recollections, usually in the form of a tape-recorded interview 2. the gathering and preservation of such data
o·ral·ism (ôr'əl iz'm) *n.* the theory or practice of teaching the deaf to read lips and to speak —**o'ral·ist** *adj., n.*
O·ran (ō ran'; *Fr.* ô rän') seaport in N Algeria, on the Mediterranean: pop. 430,000
o·rang (ō raŋ', ə-) *n. same as* ORANGUTAN
Or·ange[1] (ôr'inj, är'-) ruling family of the Netherlands: see NASSAU —*adj.* of or having to do with Orangemen
Or·ange[2] (ôr'inj, är'-; *also, for 3 & 4, Fr.* ô räⁿzh') 1. [prob. after the *orange* groves there] city in SW Calif.: suburb of Los Angeles: pop. 92,000 2. river in South Africa, flowing from NE Lesotho west into the Atlantic: c. 1,300 mi. 3. former principality of W Europe, now in SE France 4. city in SE France: pop. 21,000
or·ange (ôr'inj, är'-) *n.* [ME. < OFr. *orenge* < Pr. *auranja* (with sp. influenced by L. *aurum*, gold & loss of initial *n* through faulty separation of art. *un*) < Sp. *naranja* < Ar. *nāranj* < Per. *nārang* < Sans. *naranga*, prob. akin to Tamil *naru*, fragrant] 1. a reddish-yellow, round, edible citrus fruit, with a sweet, juicy pulp 2. any of various evergreen trees (genus *Citrus*) of the rue family producing this fruit, having white, fragrant blossoms, often carried by brides, and hard, yellow wood 3. any of several plants or fruits resembling the orange 4. reddish yellow —*adj.* 1. reddish-yellow 2. made with or from orange 3. having a flavor like that of oranges —**or'ang·y** (-in jē) *adj.*
or·ange·ade (-ād') *n.* [Fr.: see ORANGE & -ADE] a drink made of orange juice and water, usually sweetened
Orange Free State province of South Africa, west of Lesotho: formerly a Boer republic (1854–1900) & then a Brit. colony (**Orange River Colony**, 1900–10): 49,866 sq. mi.; pop. 1,387,000; cap. Bloemfontein
☆**orange hawkweed** *same as* DEVIL'S PAINTBRUSH
Or·ange·ism (ôr'inj iz'm, är'-) *n.* the principles and practices of the Orangemen
Or·ange·man (-mən) *n., pl.* **-men** (-mən) [after the Prince of *Orange*, later WILLIAM III] a member of a secret Protestant society organized in N Ireland (1795)
orang peko a black tea of Ceylon and India: see PEKOE
or·ang·ery (ôr'inj rē, är'-) *n., pl.* **-ries** [Fr. *orangerie* < *oranger*, orange tree < *orange*] a hothouse or other sheltered place for growing orange trees in cooler climates
☆**orange stick** a pointed stick, orig. of orangewood, used in manicuring

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United States Patent [19]

Potter et al.

[11] Patent Number: 6,096,320

[45] Date of Patent: Aug. 1, 2000

[54] **VACCINES WITH CHIMERIC PROTEIN COMPRISING GAMMA-INTERFERON AND LEUKOTOXIN DERIVED FROM PASTEURELLA HAEMOLYTICA**

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[73] Assignees: **University of Saskatchewan**, Saskatoon; **Ciba-Geigy Canada Ltd.**, Mississauga, both of Canada

[21] Appl. No.: **08/954,418**

[22] Filed: **Oct. 20, 1997**

Related U.S. Application Data

[60] Continuation of application No. 08/681,479, Jul. 22, 1996, abandoned, which is a division of application No. 08/170,126, Dec. 20, 1993, Pat. No. 5,594,107, which is a continuation-in-part of application No. 07/777,715, Oct. 16, 1991, Pat. No. 5,273,889, which is a continuation-in-part of application No. 07/571,301, Aug. 22, 1990, Pat. No. 5,238,823.

[51] **Int. Cl.**[7] **A61K 39/102**

[52] **U.S. Cl.** **424/255.1**; 424/192.1; 424/193.1; 424/195.11; 424/85.1; 424/85.4; 424/85.5; 435/69.5; 435/69.7; 530/350; 530/351

[58] **Field of Search** 424/255.1, 85.1, 424/192.1, 193.1, 195.11, 85.4, 85.5; 435/69.5, 69.7; 530/350, 351

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[57] **ABSTRACT**

New chimeric proteins, DNA encoding the same, and the use of these proteins in stimulating immunity against respiratory diseases such as pneumonia, including shipping fever pneumonia, are disclosed. The chimeric proteins include at least one epitope of an RTX cytotoxin fused to an active fragment of a cytokine. The chimeric proteins can be used in a vaccine composition. Also disclosed are methods of vaccination as well as methods of making the proteins employed in the vaccines.

13 Claims, 29 Drawing Sheets

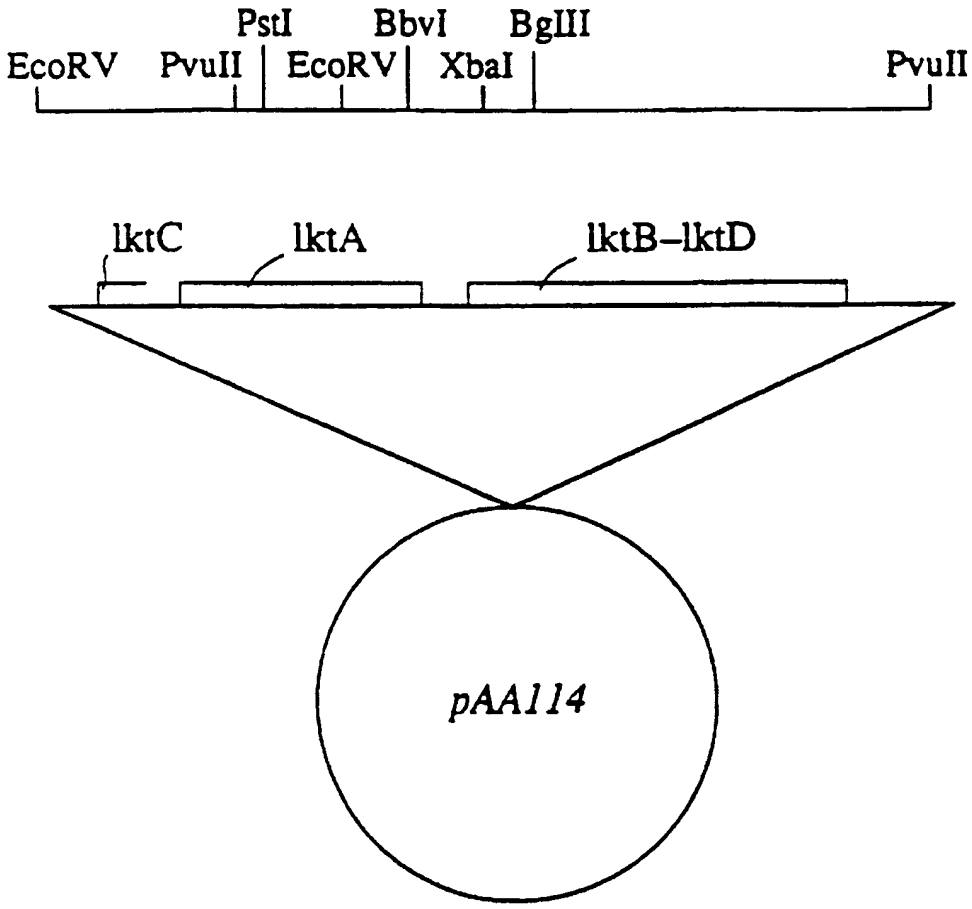

PstI
BbvI
BglII
EcoRV
PvuII
EcoRV
XbaI
PvuII
lktC
lktA
lktB–lktD
pAA114

FIG. 1

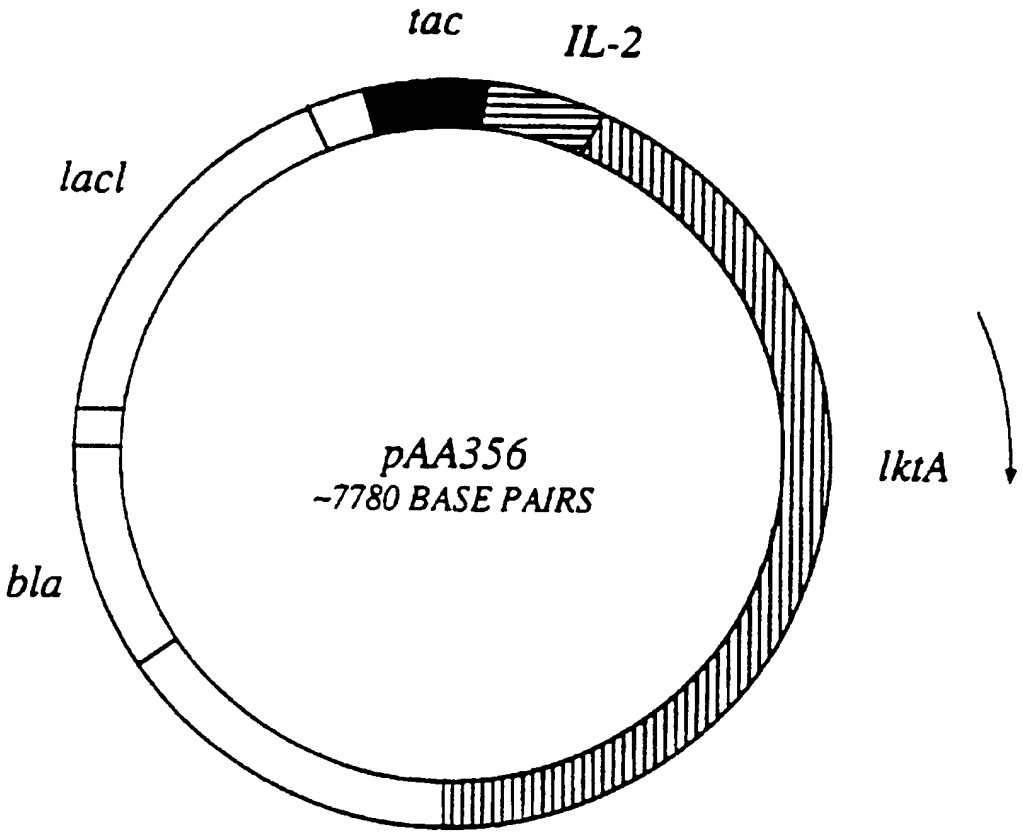


FIG. 2

```
          10          20          30          40
     *    *    *      *    *      *    *      *    *
ATG GCT ACT GTT AAT AGA TCT GCA CCT ACT TCA AGC TCT ACG GGG AAC
TAC CGA TGA CAA TTA TCT AGA CGT GGA TGA AGT TCG AGA TGC CCC TTG
Met Ala Thr Val Asn Arg Ser Ala Pro Thr Ser Ser Ser Thr Gly Asn>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

50          60          70          80          90
 *     *    *     *     *     *     *     *     *     *
ACA ATG AAA GAA GTG AAG TCA TTG CTG CTG GAT TTA CAG TTG CTT TTG
TGT TAC TTT CTT CAC TTC AGT AAC GAC GAC CTA AAT GTC AAC GAA AAC
Thr Met Lys Glu Val Lys Ser Leu Leu Leu Asp Leu Gln Leu Leu Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

  100         110         120         130         140
    *     *     *     *     *     *     *     *     *
GAG AAA GTT AAA AAT CCT GAG AAC CTC AAG CTC TCC AGG ATG CAT ACA
CTC TTT CAA TTT TTA GGA CTC TTG GAG TTC GAG AGG TCC TAC GTA TGT
Glu Lys Val Lys Asn Pro Glu Asn Leu Lys Leu Ser Arg Met His Thr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

    150         160         170         180         190
*     *     *     *     *     *     *     *     *     *
TTT GAC TTT TAC GTG CCC AAG GTT AAC GCT ACA GAA TTG AAA CAT CTT
AAA CTG AAA ATG CAC GGG TTC CAA TTG CGA TGT CTT AAC TTT GTA GAA
Phe Asp Phe Tyr Val Pro Lys Val Asn Ala Thr Glu Leu Lys His Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3A

```
      200         210         220         230         240
  *     *     *     *     *     *     *     *     *     *
AAG TGT TTA CTA GAA GAA CTC AAA CTT CTA GAG GAA GTG CTA AAT TTA
TTC ACA AAT GAT CTT CTT GAG TTT GAA GAT CTC CTT CAC GAT TTA AAT
Lys Cys Leu Leu Glu Glu Leu Lys Leu Leu Glu Glu Val Leu Asn Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

          250         260         270         280
     *     *     *     *     *     *     *     *     *
GCT CCA AGC AAA AAC CTG AAC CCC AGA GAG ATC AAG GAT TCA ATG GAC
CGA GGT TCG TTT TTG GAC TTG GGG TCT CTC TAG TTC CTA AGT TAC CTG
Ala Pro Ser Lys Asn Leu Asn Pro Arg Glu Ile Lys Asp Ser Met Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

290         300         310         320         330
  *     *     *     *     *     *     *     *     *     *
AAT ATC AAG AGA ATC GTT TTG GAA CTA CAG GGA TCT GAA ACA AGA TTC
TTA TAG TTC TCT TAG CAA AAC CTT GAT GTC CCT AGA CTT TGT TCT AAG
Asn Ile Lys Arg Ile Val Leu Glu Leu Gln Gly Ser Glu Thr Arg Phe>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

  340         350         360         370         380
     *     *     *     *     *     *     *     *     *
ACA TGT GAA TAT GAT GAT GCA ACA GTA AAC GCT GTA GAA TTT CTG AAC
TGT ACA CTT ATA CTA CTA CGT TGT CAT TTG CGA CAT CTT AAA GAC TTG
Thr Cys Glu Tyr Asp Asp Ala Thr Val Asn Ala Val Glu Phe Leu Asn>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

    390         400         410         420         430
  *     *     *     *     *     *     *     *     *     *
AAA TGG ATT ACC TTT TGT CAA AGC ATC TAC TCA ACA ATG ACT GGG GAT
TTT ACC TAA TGG AAA ACA GTT TCG TAG ATG AGT TGT TAC TGA CCC CTA
Lys Trp Ile Thr Phe Cys Gln Ser Ile Tyr Ser Thr Met Thr Gly Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      440         450         460         470         480
  *     *     *     *     *     *     *     *     *     *
CTA AGC TTC CCT AGA CTT ACA ACC CTA TCA AAT GGG CTA AAA AAC ACT
GAT TCG AAG GGA TCT GAA TGT TGG GAT AGT TTA CCC GAT TTT TTG TGA
Leu Ser Phe Pro Arg Leu Thr Thr Leu Ser Asn Gly Leu Lys Asn Thr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

          490         500         510         520
     *     *     *     *     *     *     *     *     *
TTA ACG GCA ACC AAA AGT GGC TTA CAT AAA GCC GGT CAA TCA TTA ACC
AAT TGC CGT TGG TTT TCA CCG AAT GTA TTT CGG CCA GTT AGT AAT TGG
Leu Thr Ala Thr Lys Ser Gly Leu His Lys Ala Gly Gln Ser Leu Thr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3B

```
530          540          550          560          570
  *    *    *    *    *    *    *    *    *    *    *
CAA GCC GGC AGT TCT TTA AAA ACT GGG GCA AAA AAA ATT ATC CTC TAT
GTT CGG CCG TCA AGA AAT TTT TGA CCC CGT TTT TTT TAA TAG GAG ATA
Gln Ala Gly Ser Ser Leu Lys Thr Gly Ala Lys Lys Ile Ile Leu Tyr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

  580          590          600          610          620
    *    *    *    *    *    *    *    *    *    *    *
ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT AAT GGT TTA CAG
TAA GGG GTT TTA ATG GTT ATA CTA TGA CTT GTT CCA TTA CCA AAT GTC
Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly Asn Gly Leu Gln>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

    630          640          650          660          670
*    *    *    *    *    *    *    *    *    *    *    *
GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG GTA CAA AGA GAA
CTA AAT CAG TTT CGC CGG CTT CTC AAC CCC TAA CTC CAT GTT TCT CTT
Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

       680          690          700          710          720
  *    *    *    *    *    *    *    *    *    *    *    *
GAA CGC AAT AAT ATT GCA ACA GCT CAA ACC AGT TTA GGC ACG ATT CAA
CTT GCG TTA TTA TAA CGT TGT CGA GTT TGG TCA AAT CCG TGC TAA GTT
Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu Gly Thr Ile Gln>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

          730          740          750          760
     *    *    *    *    *    *    *    *    *    *
ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA TCC GCT CCA CAA
TGG CGA TAA CCG AAT TGA CTC GCA CCG TAA CAC AAT AGG CGA GGT GTT
Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

770          780          790          800          810
  *    *    *    *    *    *    *    *    *    *    *
ATT GAT AAA TTG CTA CAG AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT
TAA CTA TTT AAC GAT GTC TTT TGA TTT CGT CCG GTT CGT AAT CCA AGA
Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3C

```
  820         830         840         850         860
    *     *     *     *     *     *     *     *     *
GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT
CGG CTT TCG TAA CAT GTT TTA CGT TTA TTT CGG TTT TGA CAT AAT AGA
Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

    870         880         890         900         910
*     *     *     *     *     *     *     *     *     *
GGC ATT CAA TCT ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT
CCG TAA GTT AGA TAA AAT CCG AGT CAT AAC CGA CCT TAC CTA AAT CTA
Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

       920         930         940         950         960
  *     *     *     *     *     *     *     *     *     *
GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC
CTC CGG AAT GTC TTA TTG TCG TTG GTT GTA CGA GAA CGA TTT CGA CCG
Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

         970         980         990         1000
    *     *     *     *     *     *     *     *     *
TTG GAG CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA
AAC CTC GAT TGT TTA AGT AAT TAA CTT TTA TAA CGA TTA AGT CAT TTT
Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

1010        1020        1030        1040        1050
  *     *     *     *     *     *     *     *     *     *
ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA
TGT GAA CTG CTT AAA CCA CTC GTT TAA TCA GTT AAA CCA AGT TTT GAT
Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 1060        1070        1080        1090        1100
    *     *     *     *     *     *     *     *     *
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC
GTT TTA TAG TTT CCG AAT CCC TGA AAT CCT CTG TTT GAG TTT TTA TAG
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   1110        1120        1130        1140        1150
*     *     *     *     *     *     *     *     *     *
GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA GGG CTA
CCA CCT GAA CTA TTT CGA CCG GAA CCA AAT CTA CAA TAG AGT CCC GAT
Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser Gly Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3D

```
      1160         1170         1180         1190         1200
  *    *    *    *    *    *    *    *    *    *    *    *
TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA
AAT AGC CCG CGT TGT CGA CGT GAA CAT GAA CGT CTA TTT TTA CGA AGT
Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

         1210         1220         1230         1240
     *    *    *    *    *    *    *    *    *    *
ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA AAC CAA GTT GTT
TGT CGA TTT TTT CAC CCA CGC CCA AAA CTT AAC CGT TTG GTT CAA CAA
Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala Asn Gln Val Val>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

1250         1260         1270         1280         1290
  *    *    *    *    *    *    *    *    *    *    *
GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA GCC CAA CGT GTT
CCA TTA TAA TGG TTT CGG CAA AGA AGA ATG TAA AAT CGG GTT GCA CAA
Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala Gln Arg Val>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 1300         1310         1320         1330         1340
    *    *    *    *    *    *    *    *    *    *
GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT GCT TTA ATT GCT TCT
CGT CGT CCA AAT AGA AGT TGA CCC GGA CAC CGA CGA AAT TAA CGA AGA
Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala Leu Ile Ala Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   1350         1360         1370         1380         1390
*    *    *    *    *    *    *    *    *    *    *    *
ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC GGT ATT GCC GAT
TGA CAA AGA GAA CGC TAA TCG GGT AAT CGT AAA CGG CCA TAA CGG CTA
Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly Ile Ala Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      1400         1410         1420         1430         1440
  *    *    *    *    *    *    *    *    *    *    *    *
AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT TAT GCC GAA CGC TTT AAA
TTT AAA TTA GTA CGT TTT TCA AAT CTC TCA ATA CGG CTT GCG AAA TTT
Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala Glu Arg Phe Lys>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3E

```
          1450          1460          1470          1480
      *     *     *     *     *     *     *     *     *
AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA TAT CAG CGG GGA
TTT AAT CCG ATA CTG CCT CTA TTA AAT AAT CGT CTT ATA GTC GCC CCT
Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

1490          1500          1510          1520          1530
  *     *     *     *     *     *     *     *     *     *
ACA GGG ACT ATT GAT GCA TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC
TGT CCC TGA TAA CTA CGT AGC CAA TGA CGT TAA TTA TGG CGT AAC CGG
Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn Thr Ala Leu Ala>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 1540          1550          1560          1570          1580
    *     *     *     *     *     *     *     *     *     *
GCT ATT GCT GGT GGT GTG TCT GCT GCT GCA GCC GGC TCG GTT ATT GCT
CGA TAA CGA CCA CCA CAC AGA CGA CGA CGT CGG CCG AGC CAA TAA CGA
Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala>
___a___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   1590          1600          1610          1620          1630
*     *     *     *     *     *     *     *     *     *
TCA CCG ATT GCC TTA TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG
AGT GGC TAA CGG AAT AAT CAT AGA CCC TAA TGG CCA CAT TAA AGA TGC
Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr>
___a___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      1640          1650          1660          1670          1680
  *     *     *     *     *     *     *     *     *     *
ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC GTT GCA AAT AAA
TAA GAC GTT ATA AGA TTT GTT CGT TAC AAA CTC GTG CAA CGT TTA TTT
Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His Val Ala Asn Lys>
___a___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

          1690          1700          1710          1720
      *     *     *     *     *     *     *     *     *
ATT CAT AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT CAC GGT AAG AAC
TAA GTA TTG TTT TAA CAT CTT ACC CTT TTT TTA TTA GTG CCA TTC TTG
Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn>
___a___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

1730          1740          1750          1760          1770
  *     *     *     *     *     *     *     *     *     *
TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG AAT TTA CAA GAT
ATG AAA CTT TTA CCA ATG CTA CGG GCA ATA GAA CGC TTA AAT GTT CTA
Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp>
___a___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3F

```
 1780         1790         1800         1810         1820
    *    *    *    *    *    *    *    *    *    *
AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG GCA GAA CGT
TTA TAC TTT AAG AAT GAC TTG AAT TTG TTT CTC AAT GTC CGT CTT GCA
Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu Arg>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   1830         1840         1850         1860         1870
*    *    *    *    *    *    *    *    *    *    *    *
GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT GAT TTA
CAG TAG CGA TAA TGA GTC GTC GTT ACC CTA TTG TTG TAA CCA CTA AAT
Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly Asp Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      1880         1890         1900         1910         1920
  *    *    *    *    *    *    *    *    *    *    *    *
GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT AAA GCC TAT
CGA CCA TAA TCG GCA AAT CCA CTT TTT CAG GAA TCA CCA TTT CGG ATA
Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

         1930         1940         1950         1960
     *    *    *    *    *    *    *    *    *    *
GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC GAT AAA TTA GTA
CAC CTA CGC AAA CTT CTT CCG TTT GTG TAA TTT CGG CTA TTT AAT CAT
Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala Asp Lys Leu Val>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

1970         1980         1990         2000         2010
  *    *    *    *    *    *    *    *    *    *    *
CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT AAT TCG GGT AAA
GTC AAC CTA AGC CGT TTG CCA TAA TAA CTA CAC TCA TTA AGC CCA TTT
Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 2020         2030         2040         2050         2060
    *    *    *    *    *    *    *    *    *    *
GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG CCA TTA TTG ACG CCG GGA
CGC TTT TGA GTC GTA TAG AAT AAG TCT TGC GGT AAT AAC TGC GGC CCT
Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu Leu Thr Pro Gly>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3G

```
   2070          2080          2090          2100          2110
*     *       *     *       *     *       *     *       *     *
ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT GAA TAT ATT ACC
TGT CTC GTA GCA CTT GCG CAT GTT TGT CCA TTT ATA CTT ATA TAA TGG
Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr Glu Tyr Ile Thr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      2120          2130          2140          2150          2160
  *     *       *     *       *     *       *     *       *     *
AAG CTC AAT ATT AAC CGT GTA GAT AGC TGG AAA ATT ACA GAT GGT GCA
TTC GAG TTA TAA TTG GCA CAT CTA TCG ACC TTT TAA TGT CTA CCA CGT
Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile Thr Asp Gly Ala>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

          2170          2180          2190          2200
      *     *       *     *       *     *       *     *
GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG CGT ATT GGT ATT
CGT TCA AGA TGG AAA CTA AAT TGA TTG CAA CAA GTC GCA TAA CCA TAA
Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln Arg Ile Gly Ile>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

2210          2220          2230          2240          2250
  *     *       *     *       *     *       *     *       *
GAA TTA GAC AAT GCT GGA AAT GTA ACT AAA ACC AAA GAA ACA AAA ATT
CTT AAT CTG TTA CGA CCT TTA CAT TGA TTT TGG TTT CTT TGT TTT TAA
Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys Glu Thr Lys Ile>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

  2260          2270          2280          2290          2300
      *     *       *     *       *     *       *     *       *
ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT GGT
TAA CGG TTT GAA CCA CTT CCA CTA CTG TTG CAT AAA CAA CCA AGA CCA
Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

    2310          2320          2330          2340          2350
  *     *       *     *       *     *       *     *       *     *
ACG ACG GAA ATT GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC
TGC TGC CTT TAA CTA CCG CCA CTT CCA ATG CTG GCT CAA GTG ATA TCG
Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      2360          2370          2380          2390          2400
  *     *       *     *       *     *       *     *       *     *
CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG
GCA CCT TTG ATA CCA CGA AAT TGA TAA CTA CGT TGG TTT CTC TGG CTC
Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3H

```
        2410        2420        2430        2440
         *   *   *   *   *   *   *   *   *
CAA GGT AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA
GTT CCA TCA ATA TGG CAT TTA GCA AAG CAT CTT TGG CCA TTT CGT GAT
Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

2450        2460        2470        2480        2490
  *   *   *   *   *   *   *   *   *   *   *   *   *
CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA
GTG CTT CAC TGA AGT TGG GTA TGG CGT AAT CAC CCG TTG GCA CTT CTT
His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 2500        2510        2520        2530        2540
    *   *   *   *   *   *   *   *   *   *   *   *   *
AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT GCC GGT TAT TAC
TTT TAT CTT ATA GCA GTA TCG TTA TTG GTC GTG GTA CGG CCA ATA ATG
Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr Tyr>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   2550        2560        2570        2580        2590
*   *   *   *   *   *   *   *   *   *   *   *   *   *
ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC GGT ACA TCA CAT
TGG TTT CTA TGG AAC TTT CGA CAA CTT CTT TAA TAG CCA TGT AGT GTA
Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr Ser His>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      2600        2610        2620        2630        2640
  *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC TTT AAC GGT GGT
TTG CTA TAG AAA TTT CCA TCA TTC AAG TTA CTA CGG AAA TTG CCA CCA
Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe Asn Gly Gly>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

        2650        2660        2670        2680
         *   *   *   *   *   *   *   *   *
GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT GAC CGC TTA TTT
CTA CCA CAG CTA TGA TAA CTG CCA TTG CTG CCG TTA CTG GCG AAT AAA
Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn Asp Arg Leu Phe>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3I

```
2690        2700        2710        2720        2730
  *     *     *     *     *     *     *     *     *     *
GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT GGT GAT GAT TTT
CCA CCA TTT CCG CTA CTA TAA GAG CTA CCA CCT TTA CCA CTA CTA AAA
Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp Phe>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 2740        2750        2760        2770        2780
    *     *     *     *     *     *     *     *     *
ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA CAC GGT GGC AAG GGC GAT
TAG CTA CCG CCA TTT CCG TTG CTG GAT AAT GTG CCA CCG TTC CCG CTA
Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly Gly Lys Gly Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   2790        2800        2810        2820        2830
*     *     *     *     *     *     *     *     *     *
GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT ATT ATT ACC GAT
CTA TAA AAG CAA GTG GCA TTT CCG CTA CCA TTA CTA TAA TAA TGG CTA
Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      2840        2850        2860        2870        2880
  *     *     *     *     *     *     *     *     *     *
TCT GAC GGC AAT GAT AAA TTA TCA TTC TCT GAT TCG AAC TTA AAA GAT
AGA CTG CCG TTA CTA TTT AAT AGT AAG AGA CTA AGC TTG AAT TTT CTA
Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser Asn Leu Lys Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

         2890        2900        2910        2920
     *     *     *     *     *     *     *     *     *
TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA
AAT TGT AAA CTT TTT CAA TTT GTA TTA GAA CAG TAG TGC TTA TCG TTT
Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile Thr Asn Ser Lys>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

2930        2940        2950        2960        2970
  *     *     *     *     *     *     *     *     *     *
AAA GAG AAA GTG ACC ATT CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT
TTT CTC TTT CAC TGG TAA GTT TTG ACC AAG GCT CTC CGA CTA AAA CGA
Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

 2980        2990        3000        3010        3020
    *     *     *     *     *     *     *     *     *
AAA GAA GTG CCT AAT TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA
TTT CTT CAC GGA TTA ATA TTT CGT TGA TTT CTA CTC TTT TAG CTT CTT
Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>
```

FIG. 3J

```
   3030          3040          3050          3060          3070
 *     *       *     *       *     *       *     *       *     *
ATC ATC GGT CAA AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT
TAG TAG CCA GTT TTA CCG CTC GCC TAG TGG AGT TTC GTT CAA CTA CTA
Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

      3080          3090          3100          3110          3120
  *     *       *     *       *     *       *     *       *     *
CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA
GAA TAG CGT TTT CCA TTG CCG TTT TAA TGG GTT CTA CTC GAT AGT TTT
Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

         3130          3140          3150          3160
     *     *       *     *       *     *       *     *     *
GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC
CAA CAA CTA TTG ATA CTT AAC GAG TTT GTA TCG TTT TTA CAC TGT TTG
Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

3170          3180          3190          3200          3210
  *     *       *     *       *     *       *     *       *     *
AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT
TCG AAT CTA TTC AAT TAG AGT AGA CAT TCA CGT AAA TGG AGC AGA TTA
Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

  3220          3230          3240          3250          3260
    *     *       *     *       *     *       *     *       *
GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT
CTA AGC TCT TTA CAT AAT CAC CGA GGT TGA AGT TAC AAC CTA GTT TCA
Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser>
___a___a___a___a___a____FUSION PROTEIN_a___a___a___a___a___a___>

   3270          3280          3290          3300      3310
 *     *       *     *       *     *       *     *     *   *
TTA TCT TCT CTT CAA TTT GCT AGG GGA TCC TAG CTAGCTAGCCATGG
AAT AGA AGA GAA GTT AAA CGA TCC CCT AGG ATC GATCGATCGGTACC
Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser End>
___a___a___a__FUSION PROTEIN___a___a___a___>
```

FIG. 3K

FIG. 4A

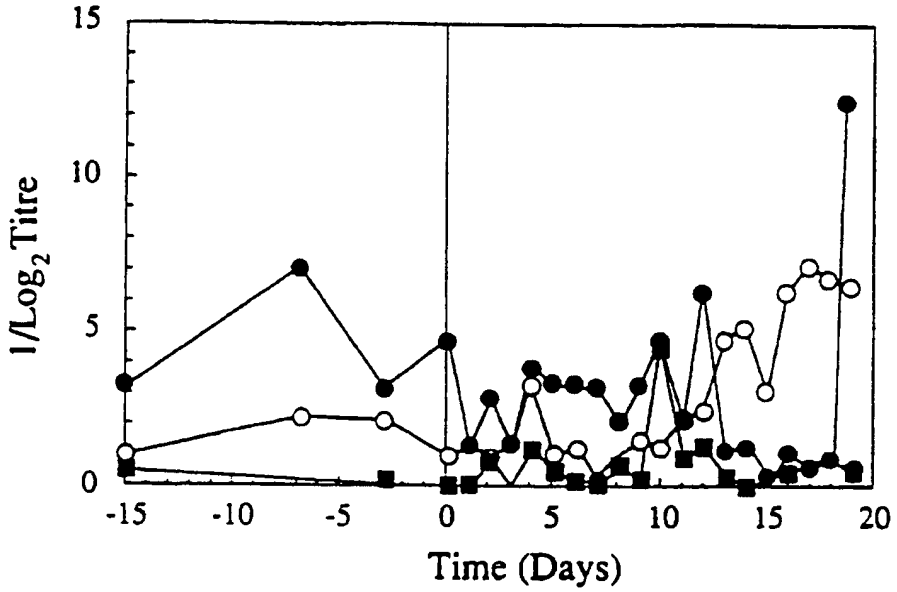

15
10
5
0
1/Log2 Titre
-15
-10
-5
0
5
10
15
20
Time (Days)

FIG. 4B

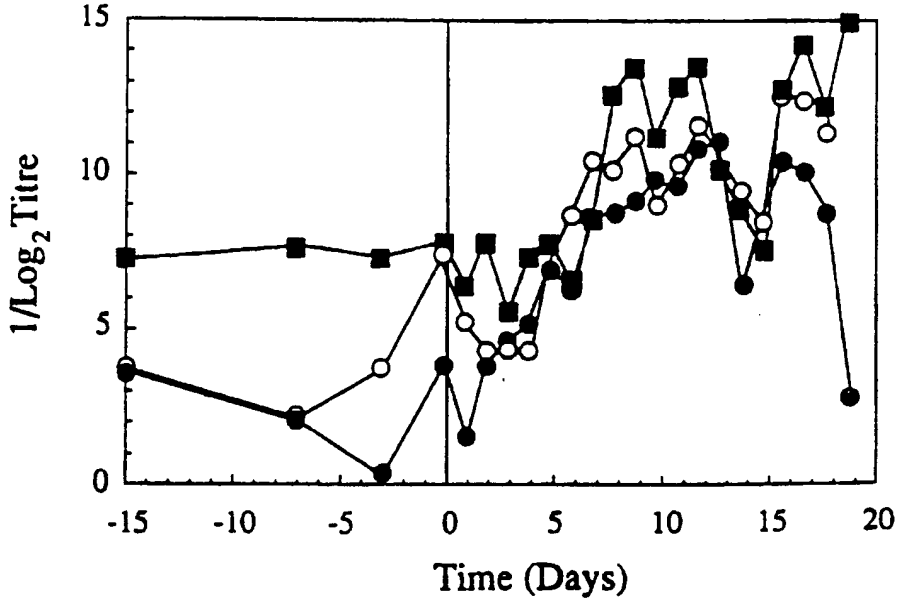
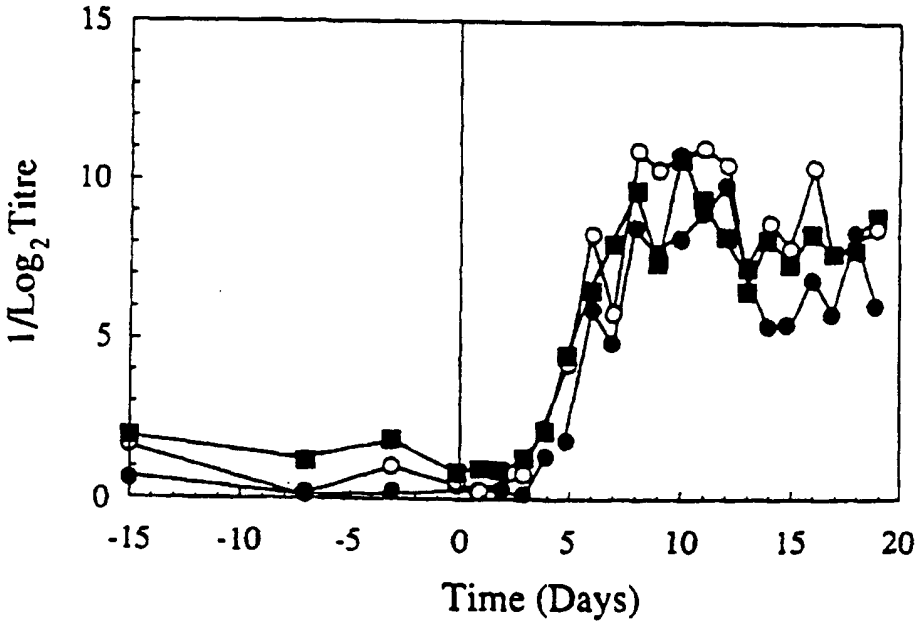

15
10
5
0
1/Log2 Titre
-15
-10
-5
0
5
10
15
20
Time (Days)

FIG. 4C



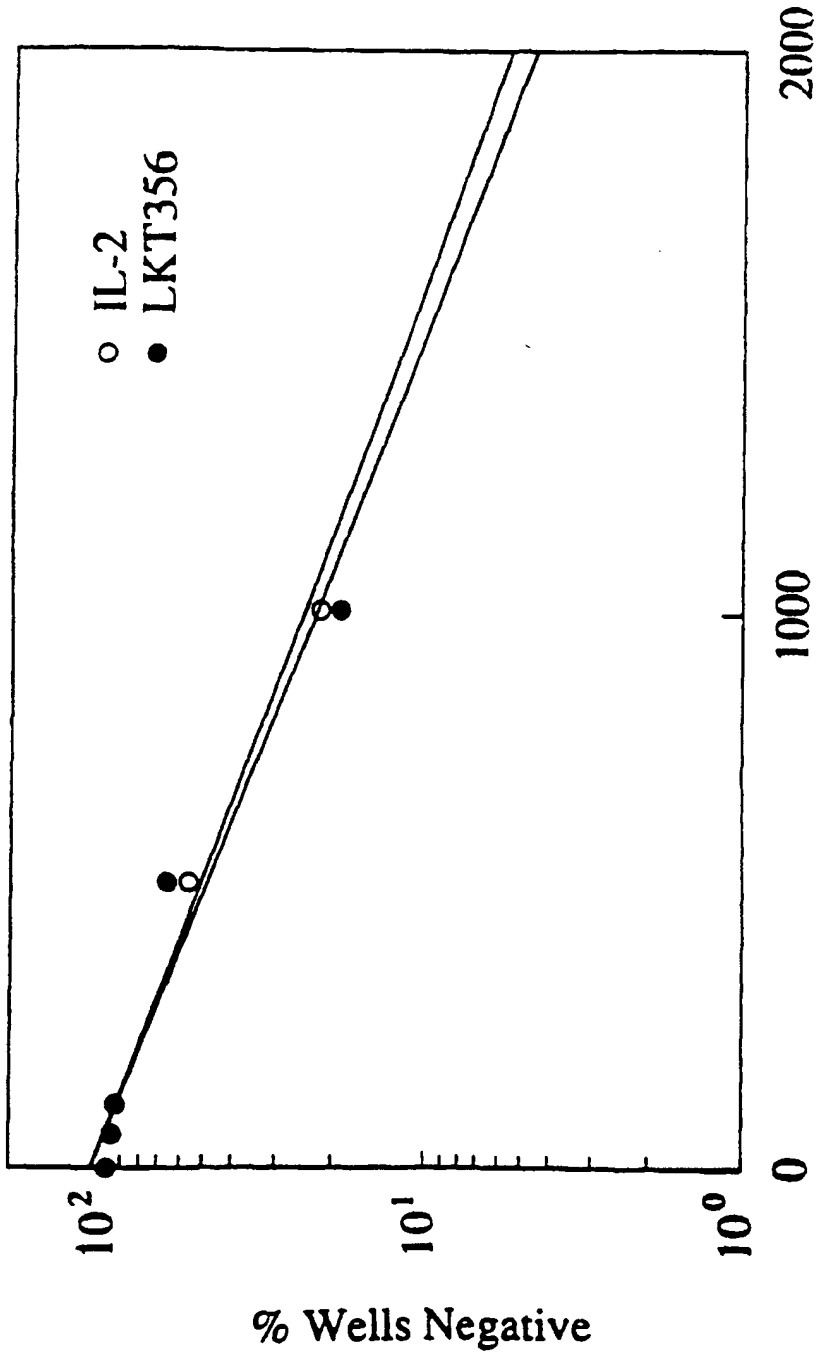

○ IL-2
● LKT356
2000
1000
0
No. of cells plated
10^2
10^1
10^0
% Wells Negative

FIG. 5

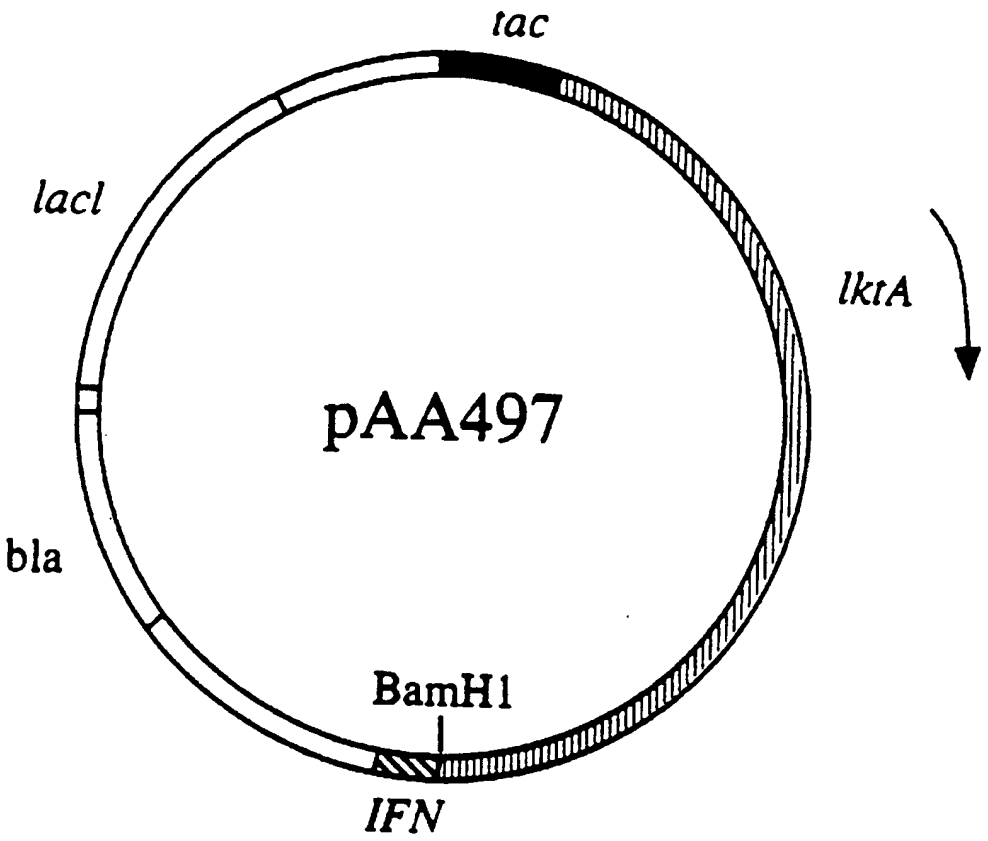


FIG. 6

```
          10          20          30          40
     *    *    *    *    *    *    *    *    *    *
ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA AAA
TAC CGA TGA CAA TAT CTA GAT TCG AAG GGT TTT TGA CCC CGT TTT TTT
Met Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys Lys>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d___10__d___d___d20__PAA352_d_30d___d___d___40__d___d___>
___a___a___VECTOR SEQUENCE_a___a___a__>

50          60          70          80          90
 *    *    *    *    *    *    *    *    *    *
ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT
TAA TAG GAG ATA TAA GGG GTT TTA ATG GTT ATA CTA TGA CTT GTT CCA
Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
50_d___d___d_60d___d___d____PAA352_d___d80_d___d___d_90d___d___>

  100         110         120         130         140
    *    *    *    *    *    *    *    *    *
AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG
TTA CCA AAT GTC CTA AAT CAG TTT CGC CGG CTT CTC AAC CCC TAA CTC
Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
__100__d___d___110_d___d____PAA352_d___d__130__d___d___140_d___>

    150         160         170         180         190
*    *    *    *    *    *    *    *    *    *
GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT CAA ACC AGT TTA
CAT GTT TCT CTT CTT GCG TTA TTA TAA CGT TGT CGA GTT TGG TCA AAT
Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d150d___d___d__160__d____PAA352_d___d___d180d___d___d__190__>
```

FIG. 7A

```
      200         210         220         230         240
  *     *     *     *     *     *     *     *     *     *
GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA
CCG TGC TAA GTT TGG CGA TAA CCG AAT TGA CTC GCA CCG TAA CAC AAT
Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___200_d___d___d210d____PAA352_20__d___d___230_d___d___d240>

          250         260         270         280
     *     *     *     *     *     *     *     *     *
TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG AAA ACT AAA GCA GGC CAA
AGG CGA GGT GTT TAA CTA TTT AAC GAT GTC TTT TGA TTT CGT CCG GTT
Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d__250__d___d___260__PAA352_d270d___d___d__280__d___d___>

290         300         310         320         330
  *     *     *     *     *     *     *     *     *     *
GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA
CGT AAT CCA AGA CGG CTT TCG TAA CAT GTT TTA CGT TTA TTT CGG TTT
Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
290_d___d___d300d___d___d___PAA352__d___320_d___d___d330d___d___>

  340         350         360         370         380
     *     *     *     *     *     *     *     *     *
ACT GTA TTA TCT GGC ATT CAA TCT ATT TTA GGC TCA GTA TTG GCT GGA
TGA CAT AAT AGA CCG TAA GTT AGA TAA AAT CCG AGT CAT AAC CGA CCT
Thr Val Leu Ser Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
__340__d___d___350_d___d____PAA352_d___d__370__d___d___380_d___>

    390         400         410         420         430
*     *     *     *     *     *     *     *     *     *
ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT
TAC CTA AAT CTA CTC CGG AAT GTC TTA TTG TCG TTG GTT GTA CGA GAA
Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d390d___d___d__400__d____PAA352_d___d___d420d___d___d__430__>

      440         450         460         470         480
  *     *     *     *     *     *     *     *     *     *
GCT AAA GCT GGC TTG GAG CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT
CGA TTT CGA CCG AAC CTC GAT TGT TTA AGT AAT TAA CTT TTA TAA CGA
Ala Lys Ala Gly Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___440_d___d___d450d____PAA352_60__d___d___470_d___d___d480>
```

FIG. 7B

```
          490          500          510          520
      *    *    *    *    *    *    *    *    *
AAT TCA GTA AAA ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT
TTA AGT CAT TTT TGT GAA CTG CTT AAA CCA CTC GTT TAA TCA GTT AAA
Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d__490__d___d___500__PAA352_d510d___d___d__520__d___d___>

530          540          550          560          570
  *    *    *    *    *    *    *    *    *    *
GGT TCA AAA CTA CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA
CCA AGT TTT GAT GTT TTA TAG TTT CCG AAT CCC TGA AAT CCT CTG TTT
Gly Ser Lys Leu Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
530_d___d___d540d___d___d___PAA352__d___560_d___d___d570d___d___>

  580          590          600          610          620
     *    *    *    *    *    *    *    *    *
CTC AAA AAT ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT
GAG TTT TTA TAG CCA CCT GAA CTA TTT CGA CCG GAA CCA AAT CTA CAA
Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
__580__d___d___590_d___d____PAA352_d___d__610__d___d___620_d___>

    630          640          650          660          670
*    *    *    *    *    *    *    *    *    *    *
ATC TCA GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT
TAG AGT CCC GAT AAT AGC CCG CGT TGT CGA CGT GAA CAT GAA CGT CTA
Ile Ser Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d630d___d___d__640__d____PAA352_d___d___d660d___d___d__670__>

      680          690          700          710          720
  *    *    *    *    *    *    *    *    *    *    *
AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA
TTT TTA CGA AGT TGT CGA TTT TTT CAC CCA CGC CCA AAA CTT AAC CGT
Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___680_d___d___d690d____PAA352_00__d___d___710_d___d___d720>

          730          740          750          760
      *    *    *    *    *    *    *    *    *
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA
TTG GTT CAA CAA CCA TTA TAA TGG TTT CGG CAA AGA AGA ATG TAA AAT
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d__730__d___d___740__PAA352_d750d___d___d__760__d___d___>
```

FIG. 7C

```
770          780          790          800          810
  *      *     *      *     *      *     *      *     *      *
 GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT GCT
 CGG GTT GCA CAA CGT CGT CCA AAT AGA AGT TGA CCC GGA CAC CGA CGA
 Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala>
  ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
770_d___d___d780d___d___d___PAA352__d___800_d___d___d810d___d___>

   820          830          840          850          860
     *      *     *      *     *      *     *      *     *
 TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC
 AAT TAA CGA AGA TGA CAA AGA GAA CGC TAA TCG GGT AAT CGT AAA CGG
 Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala>
 ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
 __820__d___d___830_d___d____PAA352_d___d__850__d___d___860_d___>

    870          880          890          900          910
  *     *      *     *      *     *      *     *      *     *
 GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT TAT GCC
 CCA TAA CGG CTA TTT AAA TTA GTA CGT TTT TCA AAT CTC TCA ATA CGG
 Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala>
 ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
 ___d870d___d___d__880__d____PAA352_d___d___d900d___d___d__910__>

       920          930          940          950          960
  *      *     *      *     *      *     *      *     *      *
 GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA
 CTT GCG AAA TTT TTT AAT CCG ATA CTG CCT CTA TTA AAT AAT CGT CTT
 Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu>
 ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
 ___d___920_d___d___d930d____PAA352_40__d___d___950_d___d___d960>

          970          980          990          1000
     *      *     *      *     *      *     *      *     *
 TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA TCG GTT ACT GCA ATT AAT
 ATA GTC GCC CCT TGT CCC TGA TAA CTA CGT AGC CAA TGA CGT TAA TTA
 Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn>
 ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
 ___d___d__970__d___d___980__PAA352_d990d___d___d_1000__d___d___>

1010         1020         1030         1040         1050
  *      *     *      *     *      *     *      *     *      *
 ACC GCA TTG GCC GCT ATT GCT GGT GGT GTG TCT GCT GCT GCA GCC GGC
 TGG CGT AAC CGG CGA TAA CGA CCA CCA CAC AGA CGA CGA CGT CGG CCG
 Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly>
  ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
1010_d___d___1020d___d___d_1_PAA352__d__1040_d___d___1050d___d___>
```

FIG. 7D

```
 1060         1070         1080         1090         1100
    *    *       *    *       *    *       *    *       *
TCG GTT ATT GCT TCA CCG ATT GCC TTA TTA GTA TCT GGG ATT ACC GGT
AGC CAA TAA CGA AGT GGC TAA CGG AAT AAT CAT AGA CCC TAA TGG CCA
Ser Val Ile Ala Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
_1060__d___d__1070_d___d____PAA352_d___d_1090__d___d__1100_d___>

   1110         1120         1130         1140         1150
*    *       *    *       *    *       *    *       *    *
GTA ATT TCT ACG ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC
CAT TAA AGA TGC TAA GAC GTT ATA AGA TTT GTT CGT TAC AAA CTC GTG
Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
___1110d___d___d_1120__d____PAA352_d___d___1140d___d___d_1150__>

      1160         1170         1180         1190         1200
  *    *       *    *       *    *       *    *       *    *
GTT GCA AAT AAA ATT CAT AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT
CAA CGT TTA TTT TAA GTA TTG TTT TAA CAT CTT ACC CTT TTT TTA TTA
Val Ala Asn Lys Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
___d__1160_d___d___1170d____PAA352_80__d___d__1190_d___d___1200>

         1210         1220         1230         1240
     *    *       *    *       *    *       *    *       *
CAC GGT AAG AAC TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG
GTG CCA TTC TTG ATG AAA CTT TTA CCA ATG CTA CGG GCA ATA GAA CGC
His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
___d___d_1210__d___d__1220__PAA352_1230d___d___d_1240__d___d___>

1250         1260         1270         1280         1290
  *    *       *    *       *    *       *    *       *    *
AAT TTA CAA GAT AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA
TTA AAT GTT CTA TTA TAC TTT AAG AAT GAC TTG AAT TTG TTT CTC AAT
Asn Leu Gln Asp Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
1250_d___d___1260d___d___d_1_PAA352__d__1280_d___d___1290d___d___>

 1300         1310         1320         1330         1340
    *    *       *    *       *    *       *    *       *
CAG GCA GAA CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC
GTC CGT CTT GCA CAG TAG CGA TAA TGA GTC GTC GTT ACC CTA TTG TTG
Gln Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE (SPLIT)_c___c___c___>
_1300__d___d__1310_d___d____PAA352_d___d_1330__d___d__1340_d___>
```

FIG. 7E

```
   1350          1360          1370          1380          1390
*     *       *     *       *     *       *     *       *     *
ATT GGT GAT TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT
TAA CCA CTA AAT CGA CCA TAA TCG GCA AAT CCA CTT TTT CAG GAA TCA
Ile Gly Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___1350d___d___d_1360__d____PAA352_d___d___1380d___d___d_1390__>

      1400          1410          1420          1430          1440
   *     *       *     *       *     *       *     *       *     *
GGT AAA GCC TAT GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC
CCA TTT CGG ATA CAC CTA CGC AAA CTT CTT CCG TTT GTG TAA TTT CGG
Gly Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d__1400_d___d___1410d____PAA352_20__d___d__1430_d___d___1440>

          1450          1460          1470          1480
      *     *       *     *       *     *       *     *     *
GAT AAA TTA GTA CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT
CTA TTT AAT CAT GTC AAC CTA AGC CGT TTG CCA TAA TAA CTA CAC TCA
Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d_1450__d___d__1460__PAA352_1470d___d___d_1480__d___d___>

1490          1500          1510          1520          1530
   *     *       *     *       *     *       *     *       *     *
AAT TCG GGT AAA GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG CCA TTA
TTA AGC CCA TTT CGC TTT TGA GTC GTA TAG AAT AAG TCT TGC GGT AAT
Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
1490_d___d___1500d___d___d_1_PAA352__d__1520_d___d___1530d___d___>

  1540          1550          1560          1570          1580
     *     *       *     *       *     *       *     *       *
TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT
AAC TGC GGC CCT TGT CTC GTA GCA CTT GCG CAT GTT TGT CCA TTT ATA
Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
_1540__d___d__1550_d___d____PAA352_d___d_1570__d___d__1580_d___>

   1590          1600          1610          1620          1630
*     *       *     *       *     *       *     *       *     *
GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT AGC TGG AAA ATT
CTT ATA TAA TGG TTC GAG TTA TAA TTG GCA CAT CTA TCG ACC TTT TAA
Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___1590d___d___d_1600__d____PAA352_d___d___1620d___d___d_1630__>
```

FIG. 7F

```
      1640        1650        1660        1670        1680
  *     *     *     *     *     *     *     *     *     *
ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG
TGT CTA CCA CGT CGT TCA AGA TGG AAA CTA AAT TGA TTG CAA CAA GTC
Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d__1640_d___d___1650d____PAA352_60__d___d__1670_d___d___1680>

        1690        1700        1710        1720
     *     *     *     *     *     *     *     *
CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA AAT GTA ACT AAA ACC AAA
GCA TAA CCA TAA CTT AAT CTG TTA CGA CCT TTA CAT TGA TTT TGG TTT
Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d_1690__d___d__1700__PAA352_1710d___d___d_1720__d___d___>

1730        1740        1750        1760        1770
  *     *     *     *     *     *     *     *     *     *
GAA ACA AAA ATT ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT
CTT TGT TTT TAA TAA CGG TTT GAA CCA CTT CCA CTA CTG TTG CAT AAA
Glu Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
1730_d___d___1740d___d___d_1_PAA352__d__1760_d___d___1770d___d___>

 1780        1790        1800        1810        1820
    *     *     *     *     *     *     *     *     *
GTT GGT TCT GGT ACG ACG GAA ATT GAT GGC GGT GAA GGT TAC GAC CGA
CAA CCA AGA CCA TGC TGC CTT TAA CTA CCG CCA CTT CCA ATG CTG GCT
Val Gly Ser Gly Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
_1780__d___d__1790_d___d____PAA352_d___d_1810__d___d__1820_d___>

   1830        1840        1850        1860        1870
*     *     *     *     *     *     *     *     *     *
GTT CAC TAT AGC CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC
CAA GTG ATA TCG GCA CCT TTG ATA CCA CGA AAT TGA TAA CTA CGT TGG
Val His Tyr Ser Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___1830d___d___d_1840__d____PAA352_d___d___1860d___d___d_1870__>

      1880        1890        1900        1910        1920
  *     *     *     *     *     *     *     *     *     *
AAA GAG ACC GAG CAA GGT AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC
TTT CTC TGG CTC GTT CCA TCA ATA TGG CAT TTA GCA AAG CAT CTT TGG
Lys Glu Thr Glu Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d__1880_d___d___1890d____PAA352_00__d___d__1910_d___d___1920>
```

FIG. 7G

```
        1930        1940        1950        1960
          *     *     *     *     *     *     *     *     *
GGT AAA GCA CTA CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC
CCA TTT CGT GAT GTG CTT CAC TGA AGT TGG GTA TGG CGT AAT CAC CCG
Gly Lys Ala Leu His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d_1930__d___d__1940__PAA352_1950d___d___d_1960__d___d___>

1970        1980        1990        2000        2010
  *     *     *     *     *     *     *     *     *     *
AAC CGT GAA GAA AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT
TTG GCA CTT CTT TTT TAT CTT ATA GCA GTA TCG TTA TTG GTC GTG GTA
Asn Arg Glu Glu Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
1970_d___d___1980d___d___d_1_PAA352__d__2000_d___d___2010d___d___>

  2020        2030        2040        2050        2060
    *     *     *     *     *     *     *     *     *
GCC GGT TAT TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC
CGG CCA ATA ATG TGG TTT CTA TGG AAC TTT CGA CAA CTT CTT TAA TAG
Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
_2020__d___d__2030_d___d____PAA352_d___d_2050__d___d__2060_d___>

   2070        2080        2090        2100        2110
  *     *     *     *     *     *     *     *     *     *
GGT ACA TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC
CCA TGT AGT GTA TTG CTA TAG AAA TTT CCA TCA TTC AAG TTA CTA CGG
Gly Thr Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___2070d___d___d_2080__d____PAA352_d___d___2100d___d___d_2110__>

      2120        2130        2140        2150        2160
  *     *     *     *     *     *     *     *     *     *
TTT AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT
AAA TTG CCA CCA CTA CCA CAG CTA TGA TAA CTG CCA TTG CTG CCG TTA
Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d__2120_d___d___2130d____PAA352_40__d___d__2150_d___d___2160>

        2170        2180        2190        2200
          *     *     *     *     *     *     *     *     *
GAC CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT
CTG GCG AAT AAA CCA CCA TTT CCG CTA CTA TAA GAG CTA CCA CCT TTA
Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn>
___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
___d___d_2170__d___d__2180__PAA352_2190d___d___d_2200__d___d___>
```

FIG. 7H

```
2210        2220        2230        2240        2250
  *     *     *     *     *     *     *     *     *     *
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 CCA CTA CTA AAA TAG CTA CCG CCA TTT CCG TTG CTG GAT AAT GTG CCA
 Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly>
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2210_d___d___2220d___d___d_2_PAA352__d__2240_d___d___2250d___d___>

  2260        2270        2280        2290        2300
    *     *     *     *     *     *     *     *     *
 GGC AAG GGC GAT GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT
 CCG TTC CCG CTA CTA TAA AAG CAA GTG GCA TTT CCG CTA CCA TTA CTA
 Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp>
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_2260__d___d__2270_d___d____PAA352_d___d_2290__d___d__2300_d___>

   2310        2320        2330        2340        2350
  *     *     *     *     *     *     *     *     *     *
 ATT ATT ACC GAT TCT GAC GGC AAT GAT AAA TTA TCA TTC TCT GAT TCG
 TAA TAA TGG CTA AGA CTG CCG TTA CTA TTT AAT AGT AAG AGA CTA AGC
 Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser>
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 ___2310d___d___d_2320__d____PAA352_d___d___2340d___d___d_2350__>

      2360        2370        2380        2390        2400
  *     *     *     *     *     *     *     *     *     *
 AAC TTA AAA GAT TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC
 TTG AAT TTT CTA AAT TGT AAA CTT TTT CAA TTT GTA TTA GAA CAG TAG
 Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile>
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 ___d__2360_d___d___2370d____PAA352_80__d___d__2390_d___d___2400>

         2410        2420        2430        2440
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 TGC TTA TCG TTT TTT CTC TTT CAC TGG TAA GTT TTG ACC AAG GCT CTC
 Thr Asn Ser Lys Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu>
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2450        2460        2470        2480        2490
  *     *     *     *     *     *     *     *     *     *
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 CGA CTA AAA CGA TTT CTT CAC GGA TTA ATA TTT CGT TGA TTT CTA CTC
 Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu>
  ___c___c____RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]_c___c___c___>
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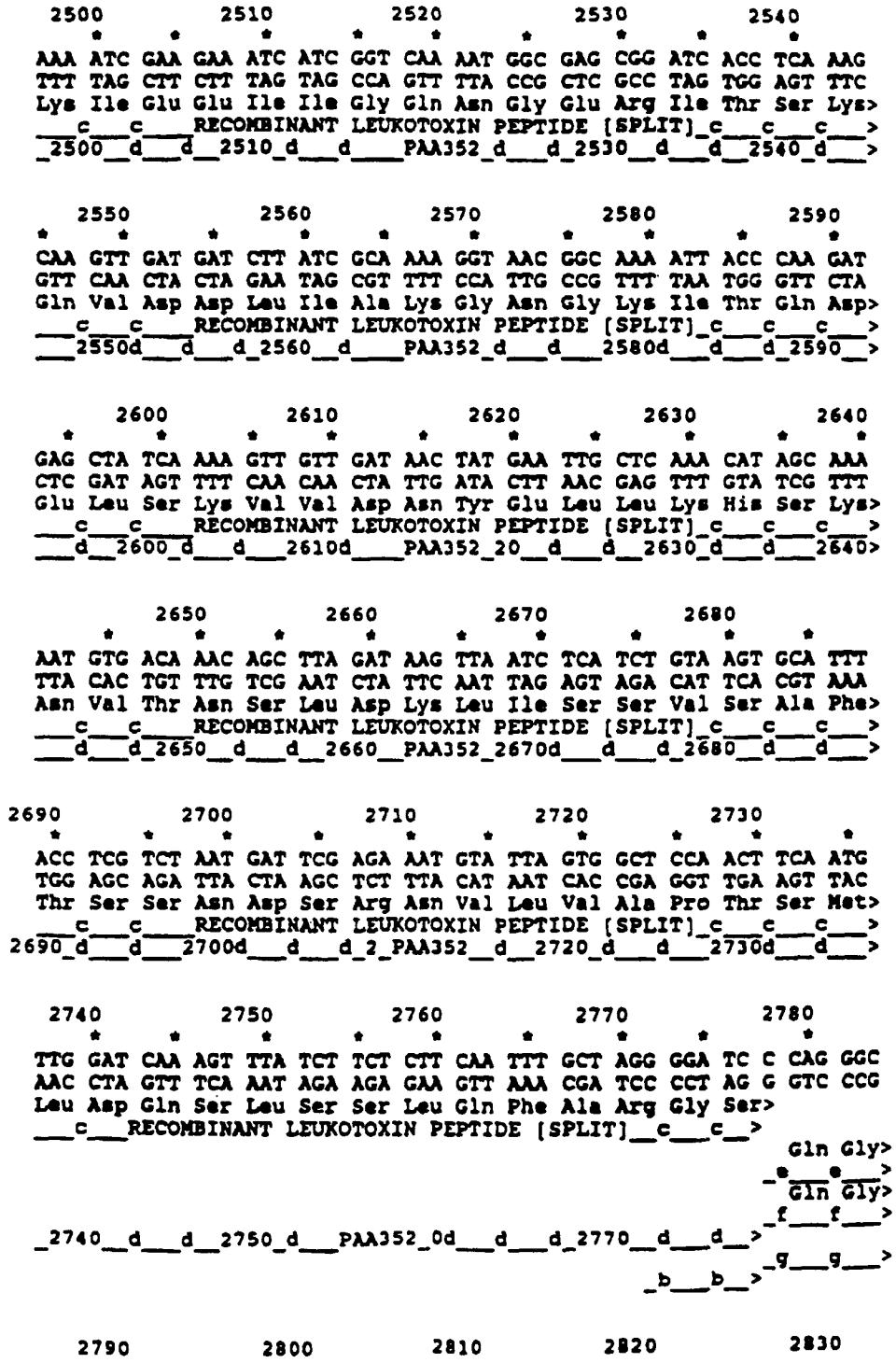
FIG. 7I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Lys Ile Glu Glu Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys>
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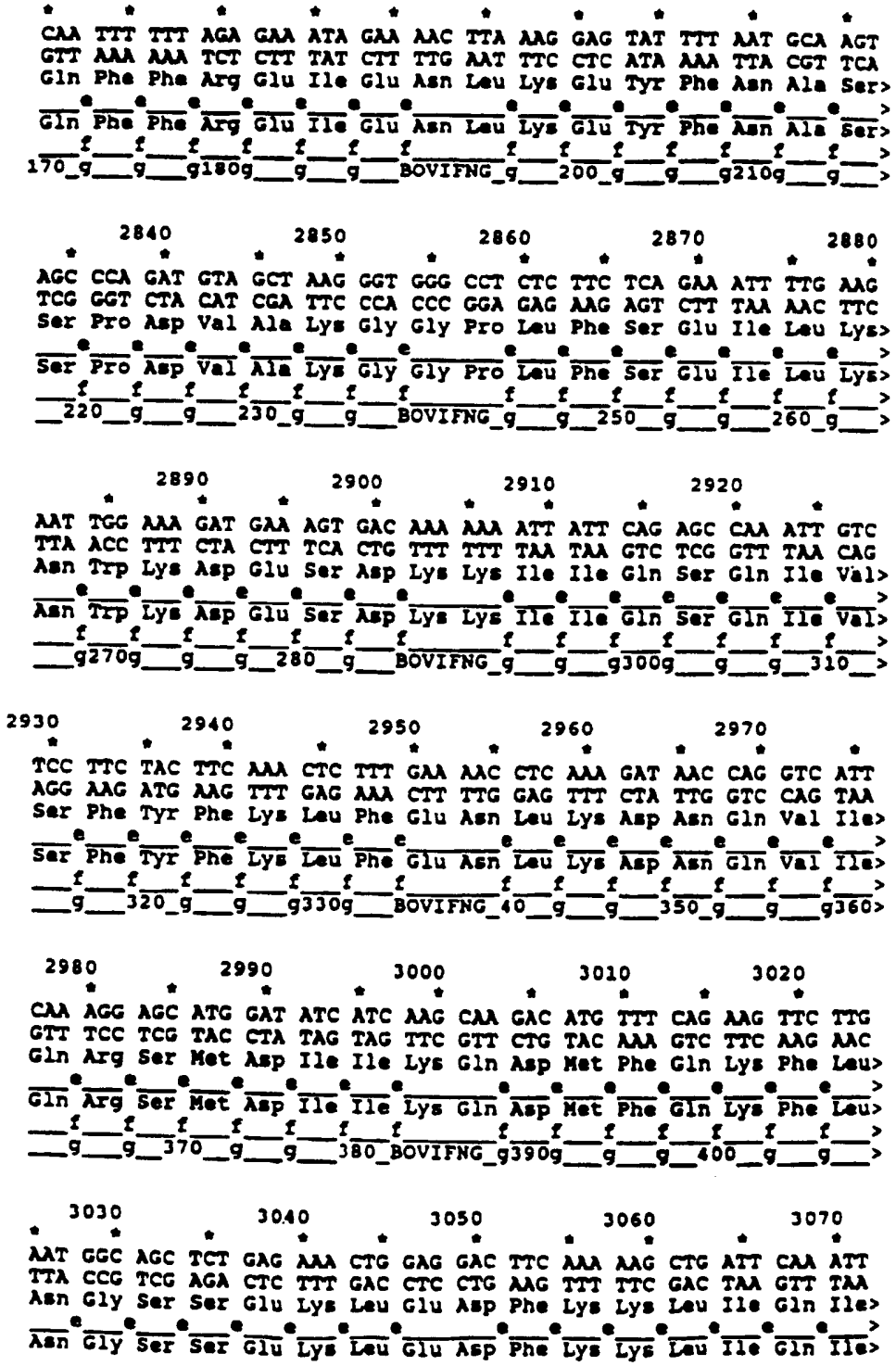
CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT
GTT CAA CTA CTA GAA TAG CGT TTT CCA TTG CCG TTT TAA TGG GTT CTA
Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp>
RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]
2600 2610 2620 2630 2640
GAG CTA TCA AAA GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA
CTC GAT AGT TTT CAA CAA CTA TTG ATA CTT AAC GAG TTT GTA TCG TTT
Glu Leu Ser Lys Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys>
RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]
2650 2660 2670 2680
AAT GTG ACA AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT
TTA CAC TGT TTG TCG AAT CTA TTC AAT TAG AGT AGA CAT TCA CGT AAA
Asn Val Thr Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe>
RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]
2690 2700 2710 2720 2730
ACC TCG TCT AAT GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG
TGG AGC AGA TTA CTA AGC TCT TTA CAT AAT CAC CGA GGT TGA AGT TAC
Thr Ser Ser Asn Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met>
RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]
2740 2750 2760 2770 2780
TTG GAT CAA AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TC C CAG GGC
AAC CTA GTT TCA AAT AGA AGA GAA GTT AAA CGA TCC CCT AG G GTC CCG
Leu Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser>
RECOMBINANT LEUKOTOXIN PEPTIDE [SPLIT]
Gln Gly>
Gln Gly>
2790 2800 2810 2820 2830

FIG. 7J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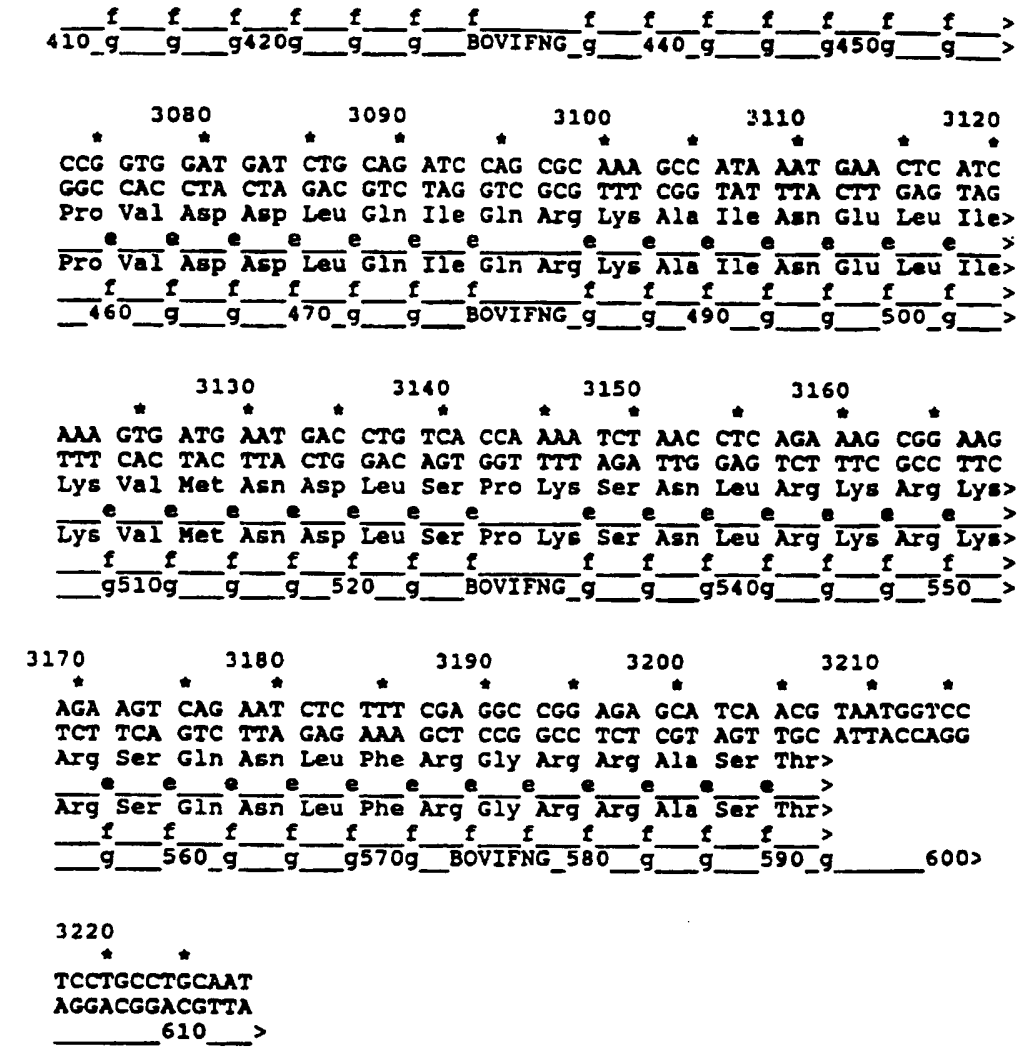
Gln Phe Phe Arg Glu Ile Glu Asn Leu Lys Glu Tyr Phe Asn Ala Ser
BOVIFNG
2840 2850 2860 2870 2880
AGC CCA GAT GTA GCT AAG GGT GGG CCT CTC TTC TCA GAA ATT TTG AAG
TCG GGT CTA CAT CGA TTC CCA CCC GGA GAG AAG AGT CTT TAA AAC TTC
Ser Pro Asp Val Ala Lys Gly Gly Pro Leu Phe Ser Glu Ile Leu Lys
2890 2900 2910 2920
AAT TGG AAA GAT GAA AGT GAC AAA AAA ATT ATT CAG AGC CAA ATT GTC
TTA ACC TTT CTA CTT TCA CTG TTT TTT TAA TAA GTC TCG GTT TAA CAG
Asn Trp Lys Asp Glu Ser Asp Lys Lys Ile Ile Gln Ser Gln Ile Val
2930 2940 2950 2960 2970
TCC TTC TAC TTC AAA CTC TTT GAA AAC CTC AAA GAT AAC CAG GTC ATT
AGG AAG ATG AAG TTT GAG AAA CTT TTG GAG TTT CTA TTG GTC CAG TAA
Ser Phe Tyr Phe Lys Leu Phe Glu Asn Leu Lys Asp Asn Gln Val Ile
2980 2990 3000 3010 3020
CAA AGG AGC ATG GAT ATC ATC AAG CAA GAC ATG TTT CAG AAG TTC TTG
GTT TCC TCG TAC CTA TAG TAG TTC GTT CTG TAC AAA GTC TTC AAG AAC
Gln Arg Ser Met Asp Ile Ile Lys Gln Asp Met Phe Gln Lys Phe Leu
3030 3040 3050 3060 3070
AAT GGC AGC TCT GAG AAA CTG GAG GAC TTC AAA AAG CTG ATT CAA ATT
TTA CCG TCG AGA CTC TTT GAC CTC CTG AAG TTT TTC GAC TAA GTT TAA
Asn Gly Ser Ser Glu Lys Leu Glu Asp Phe Lys Lys Leu Ile Gln Ile

FIG. 7K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GGC CAC CTA CTA GAC GTC TAG GTC GCG TTT CGG TAT TTA CTT GAG TAG
Pro Val Asp Asp Leu Gln Ile Gln Arg Lys Ala Ile Asn Glu Leu Ile
3130 3140 3150 3160
AAA GTG ATG AAT GAC CTG TCA CCA AAA TCT AAC CTC AGA AAG CGG AAG
TTT CAC TAC TTA CTG GAC AGT GGT TTT AGA TTG GAG TCT TTC GCC TTC
Lys Val Met Asn Asp Leu Ser Pro Lys Ser Asn Leu Arg Lys Arg Lys
3170 3180 3190 3200 3210
AGA AGT CAG AAT CTC TTT CGA GGC CGG AGA GCA TCA ACG TAATGGTCC
TCT TCA GTC TTA GAG AAA GCT CCG GCC TCT CGT AGT TGC ATTACCAGG
Arg Ser Gln Asn Leu Phe Arg Gly Arg Arg Ala Ser Thr
3220
TCCTGCCTGCAAT
AGGACGGACGTTA

FIG. 7L

VACCINES WITH CHIMERIC PROTEIN COMPRISING GAMMA-INTERFERON AND LEUKOTOXIN DERIVED FROM PASTEURELLA HAEMOLYTICA

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of application Ser. No. 08/681,479 filed on Jul. 22, 1996 now abandoned, which is a divisional of Ser. No. 08/170,126 filed on Dec. 20, 1993 now U.S. Pat. No. 5,594,107, which is a continuation-in-part of Ser. No. 07/777,715 filed on Oct. 16, 1991 (now U.S. Pat. No. 5,273,889) which is a continuation-in-part of Ser. No. 07/571,301 filed on Aug. 22, 1990 (now U.S. Pat. No. 5,238,823).

DESCRIPTION

TECHNICAL FIELD

The present invention relates generally to subunit antigens, vaccine compositions, and methods of administering the same. More particularly, the present invention relates to cytokine-cytotoxin gene fusion products and the use of the same for stimulating immunity against pneumonia.

BACKGROUND OF THE INVENTION

Respiratory disease affecting feedlot cattle causes tremendous losses yearly to the cattle industry. Calves are the most severely affected, and a large number of these calves die. This disease is associated with pathogenic microorganisms, particularly Pasteurallae species, and various stresses, such as transportation and overcrowding.

Shipping fever is the most economically important respiratory disease associated with Pasteurella species. The disease is characterized by sudden onset, usually within two weeks of stress. The symptoms include dyspnea, cough, ocular and nasal discharge, inappetence and rapid weight loss, fever, increased lung sounds, immunosuppression, general depression, viral and/or bacterial infection of the lungs. Various bacteria and viruses have been isolated from affected animals including Pasteurella spp., bovine herpes virus 1, parainfluenza-3 virus, bovine-respiratory syncytial virus and Mycoplasma species. The disease typically affects 15–30% of exposed animals and the resulting deaths are typically 2–5% of the exposed population.

Exposure of the animal to stress, plus infection with a variety of viruses, as described above, appears to make the animal susceptible to fibrinous pneumonia caused by *P. haemolytica*, and to a lesser extent, *P. multocida*. For a general background on shipping fever see Yates, W. D. G. (1982) *Can. J. Comp. Med.* 46:225–263.

P. haemolytica also causes enzootic pneumonia and can infect a wide range of animals, in addition to cattle, including economically important species such as sheep, swine, horses and fowl. *P. haemolytica* is also frequently found in the upper respiratory tract of healthy animals. Pneumonia develops when the bacteria infect the lungs of these animals. Protection against Pasteurella-associated diseases is therefore economically important to the agricultural industry.

There are two known biotypes of *P. haemolytica* designated A and T. There are also 12 recognized serotypes which have been isolated from ruminants. Biotype A, serotype 1 (referred to hereinafter as "A1") predominates in bovine pneumonia in North America. Shewen, P. E., and Wilkie, B. N. (1983) *Am. J. Vet. Res.* 44:715–719. However, antigens

isolated from different serotypes appear to be somewhat cross-reactive. See, e.g., Donanchie et al. (1984) *J. Gen. Micro.* 130:1209–1216.

Previous Pasteurellosis vaccines have utilized whole cell preparations of either live or heat killed bacteria of various serotypes as described in U.S. Pat. Nos. 4,328,210, 4,171,354, 3,328,252, 4,167,560 and 4,346,074. Traditional vaccine preparations, however, have not been effective in protecting against Pasteurella infections. Indeed, vaccinated animals are frequently more susceptible to the disease than their non-vaccinated counterparts. Martin et al. (1980) *Can. J. Comp. Med.* 44:1–10. The lack of protection offered by traditional vaccines is probably due to the absence of important antigens, virulence determinants, or the presence of immunosuppressive components in the preparations.

Other vaccine preparations have included crude supernatant extracts from *P. haemolytica*. See, e.g., Shewen, P. E., and Wilkie, B. N. (1988) in *Can. J. Vet. Res.* 52:30–36. These culture supernatants, however, contain various soluble surface antigens of the bacterium and produce variable results when administered to animals. Other preparations include capsular extracts obtained via sodium salicylate extraction (see, e.g., Donanchie et al. (1984) 130:1209–1216; U.S. Pat. No. 4,346,074), saline extracted antigens (see, e.g., Lessley et al. (1985) *Veterinary Immunology and Immunopathology* 10:279–296; Himmel et al. (1982) *Am. J. Vet. Res.* 43:764–767), and modified live Pasteurella mutants.

Still other attempts at immunization have included the use of a purified cytotoxin from *P. haemolytica*. See, e.g. Gentry et al. (1985) *Vet. Immunology and Immunopathology* 9:239–250. This cytotoxin, which is a leukotoxin, is secreted by actively growing bacteria. Shewen, P. E., and Wilkie, B. N. (1987) *Infect. Immun.* 55:3233–3236. The gene encoding this leukotoxin has been cloned and expressed in bacterial cells. Lo et al. (1985) *Infect. Immun.* 50:667–671. Calves which survive *P. haemolytica* infections possess toxin-neutralizing antibody. Cho, H. J., and Jericho, K. W. F. (1986) *Can. J. Vet. Res.* 50:27–31; Cho et al. (1984) *Can. J. Comp. Med.* 48:151–155.

Cytokines are a group of hormone-like mediators produced by leukocytes. Cytokines serve as endogenous signals that act in conjunction with antigens to amplify both localized and systemic host defense mechanisms involving macrophages, lymphocytes, and other cell types. Representative lympokines include interleukin-1 (IL1), interleukin-2 (IL2), interleukin-3 (IL3), interleukin-4 (IL4), and gamma-interferon (γIFN).

IL1 and IL2 both exhibit thymocyte mitogenic activity and IL2 stimulates T lymphocyte proliferation. IL3 stimulates the growth of hematopoietic progenitor cells and multipotential stem cells, and IL4 acts as an induction factor on resting B cells and as a B cell growth and differentiation factor. IL4 also exhibits T cell stimulatory activity.

γIFN is predominantly produced by antigen- or mitogen-stimulated T lymphocytes. γIFN has been shown to be a potent immunomodulator and appears to enhance natural killer cell activity, antibody-dependent cellular cytotoxicity, and cytotoxic T lymphocyte activity (Lawman et al. (1989) "Recombinant Cytokines and their Potential Therapeutic Value in Veterinary Medicine" in *Comprehensive Biotech, First Supplement, Animal Biotechnology*, Pages 63–106 (Pergamon Press, London).

Gene fusions provide a convenient method for the production of chimeric proteins. The expression of a chimeric protein, such as a cytokine linked to an antigenic

polypeptide, allows the simultaneous delivery of both agents to a desired recipient. PCT Publication No. WO 88/00971 (publication date of Feb. 11, 1988) describes the fusion of an IL2 gene with the influenza hemagglutinin coding sequence and the subsequent administration of the fusion protein using a viral vector. The application nowhere contemplates the use of a cytokine fused to leukotoxin for the treatment of pneumonia in animals.

DISCLOSURE OF THE INVENTION

The present invention is based on the construction of novel gene fusions between sequences encoding certain cytokines and sequences encoding a cytotoxin derived from the RTX family of toxins, such as the *P. haemolytica* leukotoxin gene. These constructs produce fusion proteins that can be used to protect cattle and other animals from a number of diseases, depending on the particular fusion, including but not limited to respiratory diseases such as *pneumonia, including shipping fever pneumonia.*

In one embodiment, the present invention is directed to a DNA construct comprising a first nucleotide sequence encoding a cytokine, or an active fragment thereof, operably linked to a second nucleotide sequence encoding at least one epitope of an RTX cytotoxin. In particularly preferred embodiments, the first nucleotide sequence encodes IL2 or γIFN, or active fragments thereof and the second nucleotide sequence encodes a leukotoxin.

In another embodiment, the subject invention is directed to expression cassettes comprised of (a) the DNA constructs above and (b) control sequences that direct the transcription of the constructs whereby the constructs can be transcribed and translated in a host cell.

In yet another embodiment, the invention is directed to host cells transformed with these expression cassettes.

Another embodiment of the invention provides a method of producing a recombinant polypeptide comprising (a) providing a population of host cells described above and (b) growing the population of cells under conditions whereby the polypeptide encoded by the expression cassette is expressed.

In still another embodiment, the invention is directed to an immunogenic chimeric protein comprising a cytokine, or an active fragment thereof, linked to at least one epitope of an RTX cytotoxin. In particularly preferred embodiments, the cytokine is derived from bovine IL2 or bovine γIFN and the RTX cytotoxin is a leukotoxin.

Also disclosed are vaccine compositions comprising the chimeric proteins and a pharmaceutically acceptable vehicle and methods of vaccinating a subject using the same.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the structure of the leukotoxin gene of *P. haemolytica* as found in plasmid pAA114.

FIG. 2 shows the structure of plasmid pAA356 carrying a bovine IL2-leukotoxin (IL2-LKT) gene fusion wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the β-lactamase gene (ampicillin resistance); lktA is the *P. haemolytica* leukotoxin structural gene; IL2 is the bovine interleukin-2 structural gene; and lacI is the *E. coli* lac operon repressor.

FIGS. 3A–3K (SEQ ID NOS:1–2) show the nucleotide sequence and predicted amino acid sequence of the bovine IL2-LKT chimeric protein from pAA356.

FIGS. 4A–4C depict the serological response to *P. haemolytica* LKT and the IL2-LKT chimeric molecule. FIG. 4A shows the changes in IgG anti-LKT in nonimmunized calves; FIG. 4B shows the changes in IgG anti-LKT in LKT-immunized calves; and FIG. 4C shows the changes in IgG anti-LKT in calves immunized with an IL2-LKT fusion protein.

FIG. 5 shows precursor frequency analysis of PBMC responding to recombinant bovine IL2-LKT chimeric protein.

FIG. 6 shows the structure of plasmid pAA497 carrying a bovine γIFN-LKT gene fusion wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the β-lactamase gene (ampicillin resistance); lktA is the *P. haemolytica* leukotoxin structural gene; IFN is the bovine gamma-interferon structural gene; and lacI is the *E. coli* lac operon repressor.

FIGS. 7A–7L (SEQ ID NOS:3–4) depict the nucleotide sequence and predicted amino acid sequence of the bovine γIFN-LKT chimeric protein from pAA497.

DETAILED DESCRIPTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); Maniatis, Fritsch & Sambrook, *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning*, Vols. I and II (D. N. Glover ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Animal Cell Culture* (R. K. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I–IV (D. M. Weir and C. C. Blackwell eds., 1986, Blackwell Scientific Publications).

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "cytokine" is meant any one of the group of hormone-like mediators produced by T and B lymphocytes. Representative cytokines include but are not limited to IL1, IL2, IL3, IL4 and γIFN. An "active" fragment of a cytokine is a fragment of a cytokine which retains activity as determined using standard in vitro and in vivo assays. For example, assays for determining IL2 and γIFN activity are described in the Examples. See also Campos, M. (1989) *Cell. Immun.* 120:259–269 and Czarniecki, C. W. (1986) *J. Interferon Res.* 6:29–37. Assays for determining the activity of other cytokines are known and can readily be conducted by those having ordinary skill in the art.

The term "RTX cytotoxin" intends a cytotoxin belonging to the family of cytolytic toxins known as the RTX proteins. The toxins are characterized by a series of repeated amino acid domains near the carboxy terminus. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO:5), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from

Pasteurella and Actinobacillus, such as those found in *P. haemolytica, Actinobacillus pleuropneumoniae, A. actinomycetemcomitans, A. suis*, as well as the cytotoxins found in *Proteus vulgaris, Morganella morganii, Moraxella bovis, Neisseria meningitidis, H. influenzae* type B, *E. coli* alpha hemolysin and *Bordetella pertussis* adenylate cyclase hemolysin. (For further descriptions of these toxins, see, e.g., Strathdee, C. A., and Lo, R. Y. C. (1987) *Infect. Immun.* 55:3233–3236; Lo, R. Y. C. (1990) *Can. J. Vet. Res.* 54:S33–S35; Welch, R. A. (1991) *Mol. Microbiol.* 5:521–528); Lo et al. (1987) *Infect. Immun.* 55:1987–1996; Glaser et al. (1988) *Molec. Microbiol.* 2:19–30; Lally et al. (1989) *J. Biol. Chem.* 254:15451–15456; Kolodrubetz et al. (1989) *Infect. Immun.* 57:1465–1469; Chang et al. (1989) *DNA* 8:635–647; Frey, J. and Nicolet, J. (1988) *Infect. Immun.* 56:2570–2575; Devenish et al. (1989) *Infect. Immun.* 57:3210–3213; Koronakis et al. (1987) *J. Bacteriol.* 169:1509–1515 and Highlander et al. (1989) *DNA* 8:15–28). The desired cytotoxin may be chemically synthesized, isolated from an organism expressing the same, or recombinantly produced.

Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native cytotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogs. Although the native full-length cytotoxins described above display cytolytic activity, the term "cytotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of the native toxins. Thus, for example, with respect to the leukotoxins described above, molecules which lack leukotoxic activity yet remain immunogenic, would be captured by the term "leukotoxin." Such a molecule is present in plasmid pAA356, described further below. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Pat. Nos. 4,957,739 and 5,055,400; Lo et al. (1985) *Infect. Immun.* 50:667–67; Lo et al. (1987) *Infect. Immun.* 55:1987–1996; Strathdee, C. A., and Lo, R. Y. C. (1987) *Infect. Immun.* 55:3233–3236; Highlander et al. (1989) *DNA* 8:15–28; Welch, R. A. (1991) *Mol. Microbiol.* 5:521–528.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." One such epitope is the consensus sequence found among the RTX family of toxins described above. This sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO:5), where X is preferably Lys, Asp, Val or Asn. Other substitutions for X in the consensus sequence are also contemplated including substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or γδ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest.

The terms "immunogenic" protein, polypeptide or amino acid sequence refer to an amino acid sequence which elicits an immunological response as described above. An "immunogenic" protein, polypeptide-or amino acid sequence, as used herein, includes the full-length (or near full-length) sequence of the protein in question, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a polypeptide which includes one or more epitopes and thus elicits the immunological response described above. Such fragments will usually be at least about 2 amino acids in length, more preferably about 5 amino acids in length, and most preferably at least about 10 to 15 amino acids in length. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes of the protein.

The term "protein" is used herein to designate a naturally occurring polypeptide. The term "polypeptide" is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from a source occurring in nature. Thus, the term "native leukotoxin" would include naturally occurring leukotoxin and fragments thereof.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "rotavirus VP6 protein" refers to the art-recognized major viral protein of the inner capsid from any species or strain within the family Reoviridae. See, e.g., Kapikian et al., 1985. Examples of rotavirus strains from which the VP6 protein can be isolated and employed in the present invention include, but are not limited to, Simian SA-11, human D rotavirus, bovine UK rotavirus, human Wa or W rotavirus, human DS-1 rotavirus, rhesus rotavirus, the "O" agent, bovine NCDV rotavirus, human S2 rotavirus, human KUN rotavirus, human 390 rotavirus, human P rotavirus, human M rotavirus, human Walk 57/14 rotavirus, human Mo rotavirus, human Ito rotavirus, human Nemoto rotavirus, human YO rotavirus, human McM2 rotavirus, rhesus monkey MMU18006 rotavirus, canine CU-1 rotavirus, feline Taka rotavirus, equine H-2 rotavirus, human St. Thomas No. 3 and No. 4 rotaviruses, human Hosokawa rotavirus, human Hochi rotavirus, porcine SB-2 rotavirus, porcine Gottfried rotavirus, porcine SB-1A rotavirus, porcine OSU rotavirus, equine H-1 rotavirus, chicken Ch.2 rotavirus, turkey Ty.1 rotavirus, bovine C486 rotavirus, and strains derived from them. Thus the present invention encompasses the use of VP6 from any rotavirus strain, whether from subgroup I, subgroup II, or any as yet unidentified subgroup, as well as from any of the serotypes 1–7, as well as any as yet unidentified serotypes. Such VP6 proteins can be used as immunologic carriers of polypeptides. These carrier molecules comprise amino acid sequences of rotavirus VP6 amino acid sequences which are unique to the class, or any member of the class, of VP6 polypeptides. Such unique sequences of VP6 proteins are referred to as a "rotavirus VP6 inner capsid protein amino acid sequence."

A carrier that is "substantially homologous to a rotavirus VP6 inner capsid protein or a functional fragment thereof" is one in which at least about 85%, preferably at least about 90%, and most preferably at least about 95%, of the amino acids match over a defined length of the molecule. A "functional fragment" of a rotavirus VP6 inner capsid pro-

tein is a fragment with the capability of acting as a carrier molecule for the novel chimeric proteins of the instant invention.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide in a host cell when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the −10 and −35 consensus sequences.

DNA "control sequences" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into MRNA, which is then translated into a chimeric polypeptide encoded by the two coding sequences. The coding sequences need not be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired chimeric protein.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra; *DNA Cloning*, vols I & II, supra; *Nucleic Acid Hybridization*, supra. A "substantially homologous" sequence also intends a sequence that encodes a protein which is functionally equivalent to the depicted sequences. By "functionally equivalent" is meant that the amino acid sequence of the subject fusion protein is one that will elicit an immunological response, as defined above, equivalent to the response elicited by the unmodified chimeric protein.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

A composition containing A is "substantially free of" B when at least about 85% by weight of the total of A+B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A+B in the composition, more preferably at least about 95%, or even 99% by weight.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms or the disease of interest (therapy).

B. General Methods

Central to the instant invention is the production of a chimeric protein comprising a cytokine and a cytotoxin belonging to the RTX family of proteins, preferably leukotoxin. This chimeric protein can be used in a vaccine composition to protect animals against a variety of diseases, including respiratory diseases such as pneumonia, including shipping fever pneumonia.

As explained above, cytotoxins contemplated for use in the instant chimeric proteins include any of the various toxins derived from the RTX family of molecules. It is to be understood that modifications of the native amino acid sequence of these toxins which result in proteins which have substantially equivalent or enhanced activity as compared to the native sequences, are also contemplated. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutation of hosts which produce the cytotoxins. All of these modifications are included, so long as immunogenic activity is retained. Additionally, both full-length cytotoxins, immunogenic fragments thereof, and fusion proteins comprising the same, are intended for use in the subject vaccines.

Particularly useful in the subject chimeric proteins are leukotoxins, and in particular, leukotoxin derived from *P. haemolytica*. The sequence of the various full-length RTX leukotoxins are known and have been described (see, e.g., U.S. Pat. Nos. 4,957,739 and 5,055,400; Lo et al. (1985) *Infect. Immun.* 50:667–67; Lo et al. (1987) *Infect. Immun.* 55:1987–1996; Strathdee, C. A., and Lo, R. Y. C. (1987)

Infect. Immun. 55:3233–3236; Highlander et al. (1989) *DNA* 8:15–28; Welch, R. A. (1991) *Mol. Microbiol.* 5:521–528. However, useful leukotoxins include both full-length and truncated forms of the molecule which eliminate the cytotoxic activity thereof. For example, the truncated leukotoxin, LKT 352, derived from the lktA gene present in plasmid pAA352 (ATCC Accession No. 68283), and found in plasmid pAA497 (described further below), will find use in the present chimeras. The cloning strategy for this leukotoxin is described in the examples herein as well as in International Publication No. W091/15237. LKT 352 is a leukotoxin having 931 amino acids, which lacks the cytotoxic portion of the molecule. of course, the gene encoding LKT 352 need not be physically derived from the sequence present in plasmid pAA352. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, sequence variations may be present so long as the protein is immunogenic.

Similarly, the coding sequences for numerous cytokines have been elucidated. See, e.g., published EPA 088,622 and EPA 230,119 (both describing sequences for bovine γIFN); Maliszewski et al. (1988) *Molec. Immun.* 25:429–437 and Ceretti et al. (1986) *Proc. Natl. Acad. Sci.*, U.S.A. 83:2332–2337. Again, these cytokines can be purified using standard techniques.

Purification of the above proteins permits the sequencing of the same by any of the various methods known to those skilled in the art. For example, the amino acid sequences of the subject proteins can be determined from the purified proteins by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art. Furthermore, fragments of the proteins can be tested for biological activity and active fragments used in compositions in lieu of the entire protein.

Once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen DNA libraries for genes encoding the subject proteins or for analogous genes in related species. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*: Vol. I, supra; *Nucleic Acid Hybridization*, supra; *Oligonucleotide Synthesis*, supra; Sambrook et al., supra.

First, a DNA library is prepared. The library can consist of a genomic DNA library from the bacteria of interest (for the isolation of the cytotoxin gene) or from appropriate T cells (for the isolation of the desired cytokine gene). Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the gene encoding the desired protein. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as to correspond to the codons encoding a known amino acid sequence from the desired protein. Since the genetic code is degenerate, it will often be necessary to synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. In certain circumstances, it may be desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein of interest. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment. Automated oligonucleotide synthesis has made the preparation of large families of probes relatively straightforward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an evolutionarily close or distant species. The selection of the appropriate conditions is within the skill of the art. See, generally, *Nucleic Acid hybridization*, supra. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a sufficient degree of nucleic acid homology (e.g., at least about 75%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a gene for the desired protein.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), YCp19 (Saccharomyces) and bovine papilloma virus (mammalian cells). See, Generally, *DNA Cloning*: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

Suitable restriction enzymes can then be employed to isolate the appropriate cytokine gene or cytotoxin gene and these sequences can be ligated together, using standard techniques (see, e.g., Sambrook et al., supra) and cloned to form a cytokine-cytotoxin fusion gene. It has been found that the cytokine gene can be fused either 5' or 3' to the particular cytotoxin gene in question. For example, the IL2-leukotoxin fusion described in the examples includes the IL2 gene fused to the 5'-end of the full-length lktA

leukotoxin gene, whereas the γIFN-leukotoxin fusion includes the γIFN gene linked to the 3'-end of the truncated lktA gene.

The fusion gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the chimeric protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The chimeric proteins of the present invention can be expressed using, for example, native *P. haemolytica* promoter, the *E. coli* tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Pat. Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular fusion coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular chimeric protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the chimeric proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; *DNA Cloning*, Vols. I and II, supra; *Nucleic Acid Hybridization*, supra.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Pat. Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Pat. Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The chimeric protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform an appropriate microorganism and pooling and screening individual colonies using polyclonal serum or monoclonal antibodies to the desired antigen.

The chimeric proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The proteins of the present invention or their fragments can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of the present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling et al., *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Pat. Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the antigen of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against.

Animals can be immunized with the compositions of the present invention by administration of the chimeric protein, or a fragment thereof, or an analog thereof. The chimeric protein can consist of an epitope of an RTX cytotoxin fused to an active fragment of a cytokine, as defined above. Thus, if the fragment or analog of the fusion protein is used, it will include the amino acid sequence of an epitope of the desired cytotoxin which interacts with the immune system to immunize the animal to that and structurally similar epitopes, and an active fragment of a cytokine as defined above.

Chimeric proteins used to immunize a subject contain at least 6–30 amino acids which form the sequence of the desired chimeric protein, and include a cytotoxin epitope and an active cytokine fragment.

Prior to immunization, it may be desirable to increase the immunogenicity of the particular chimeric protein, or an analog of the protein, or particularly fragments of the protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the antigenic peptide may be administered linked to a carrier. For example, a fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles. Especially usefusubsotein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the chimeric proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in allowed U.S. patent application Ser. No. 07/489,790, filed Mar. 2, 1990, and incorporated herein by reference. Also useful is a fusion product of a viral protein and the subject cytokine-cytotoxin immunogen made by methods disclosed in U.S. Pat. No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the fusion proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

The novel chimeric proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel chimeric proteins can be constructed as follows. The DNA encoding the particular cytokine-cytotoxin chimeric protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant chimeric protein into the viral genome. The resulting TK-recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

It is also possible to immunize a subject with a protein of the present invention, or a protective fragment thereof, or an analog thereof, which is administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., 15th edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the individual being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the chimeric protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The chimeric proteins can also be delivered using implanted mini-pumps, well known in the art.

Furthermore, the chimeric proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as

isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, the polypeptide of interest, or an immunologically active fragment thereof, is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal delivery, are also acceptable. Injectable vaccine formulations will contain an effective amount of the active ingredient in a vehicle, the exact amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. With the present vaccine formulations, 50 μg of active ingredient per ml of injected solution should be adequate to raise an immunological response when a dose of 1 to 5 ml per animal is administered. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the particular antigen or fragment thereof, or analog thereof, in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity to pneumonia.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

Strain	Deposit Date	ATCC No.
P. haemolytica serotype 1 B122	February 1, 1989	53863
pAA356 in *E. coli* W1485	August 14, 1990	68386
pAA352 in *E. coli* W1485	March 30, 1990	68283

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T^4 DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

CDNA and genomic libraries were prepared by standard techniques in pUC13 and the bacteriophage lambda gt11, respectively. See *DNA Cloning*: Vols I and II, supra.

P. haemolytica biotype A, serotype 1 ("A1") strain B122 was isolated from the lung of a calf which died of pneumonic pasteurellosis and was stored at −70° C. in defibrinated blood. Routine propagation was carried out on blood agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% (v/v) horse serum (Gibco Canada Ltd., Burlington, Canada). All cultures were incubated at 37° C.

EXAMPLE 1

Construction of an IL2-leukotoxin Gene Fusion

1. Modification of the Bovine IL2 Gene

The bovine IL2 gene, in the plasmid pBOVIL2 (CIBA-GEIGY, Basel, Switzerland), was digested to completion with the restriction endonuclease BclI and the single-stranded ends removed by Mung Bean nuclease treatment. The DNA was then digested with EcoRI in order to excise the IL2 gene fragment. This fragment was ligated into the cloning vector pTZ19R (Pharmacia, Canada) (EcoRI/SmaI-digested). Sequence analysis revealed two populations of clones which differed only in the reading frame at the 3'-end of the gene. The first, pAA284, had a terminal sequence of 5'-TCA ACA ATG ACT GGGATC CTC-3' (BamHI site in vector underlined) while the second, pAA285, had a terminal sequence of 5'-TCA ACA ATG ACT GGG GAT CCT-3'. The sequences shown in bold face are from the IL2 gene. Because of the differences in reading frame, heterologous genes in two out of three reading frames can be fused to the bovine IL2 gene.

2. Construction of IL2-LKT Fusions

To isolate the leukotoxin gene, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See Lo et al., *Infect. Immun.*, supra; *DNA Cloning*: Vols. I and II, supra; and Sambrook et al., supra. A genomic library was constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and-individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See Lo et al., *Infect. Immun.*, supra. To confirm this, smaller fragments were recloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5' to 3' direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This con-

struct contained the entire leukotoxin gene sequence. The structure of this plasmid is shown in FIG. 1.

lktA, a MaeI restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the SmaI site of the cloning vector pUC13. This plasmid was named pAA179. From this, an expression construct was made in the ptac-based vector pGH432: lacI digested with SmaI. This construct was termed pAA345 and contained the entire MaeI fragment described above. This plasmid expresses full-length leukotoxin.

The plasmid pAA345 containing the *P. haemolytica* leukotoxin gene lktA was digested with BamHI and BglII, and the 2.75 kilobase fragment was ligated into BamHI-digested pAA285 (above). The resulting plasmid, pAA354, was digested with ApaLI, the 5'-overhang filled in with the Klenow fragment of DNA polymerase I, and finally digested with BamHI. The IL2-LKT fragment was gel purified and ligated into the expression vector pGH433 lacI which had been cut with BglII, filled in with Klenow polymerase and digested with BamHI. The resulting clone, pAA356 (ATCC No. 68386), contained the IL2-LKT gene fusion under the control of the *E. coli* tac promoter. FIG. 2 shows the structure of pAA356 while FIG. 3 (SEQ ID NO:1–2) shows the nucleotide sequence and corresponding amino acid sequence of the fusion protein expressed by this plasmid. The resulting fusion is a gene fusion of bovine IL2 to the 5'-end of the full-length lktA gene (approximately 750 bp).

3. Purification of Recombinant IL2-LKT

Recombinant IL2-LKT was purified using the following procedure. Five to ten colonies of *E. coli* W1485/pAA356 (ATCC no. 68386) were inoculated into 10 ml of TB broth supplemented with 100 micrograms/ml of ampicillin and incubated at 37° C. for 6 hours on a G10 shaker, 220 rpm. Four ml of this culture was diluted into each of two baffled Fernbach flasks containing 400 ml of TB broth+ampicillin and incubated overnight as described above. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in polypropylene bottles, 500 ml volume, using a Sorvall GS3 rotor. The pellet was resuspended in an equal volume of TB broth containing ampicillin which had been prewarmed to 37° C. (i.e., 2x400 ml), and the cells were incubated for 2 hours as described above.

3.2 ml of isopropyl-B,D-thiogalactopyranoside (IPTG, Gibco/BRL), 500 mM in water (final concentration=4 mM), was added to each culture in order to induce synthesis of recombinant IL2-LKT. Cultures were incubated for two hours. Cells were harvested by centrifugation as described above, resuspended in 30 ml of 50 mM Tris-hydrochloride, 25% (w/v) sucrose, pH 8.0, and frozen at –70° C. The frozen cells were thawed at room temperature after 60 minutes at –70° C., and 5 ml of lysozyme (Sigma, 20 mg/ml in 250 mM Tris-HCl, pH 8.0) was added. The mixture was vortexed at high speed for 10 seconds and then placed on ice for 15 minutes. The cells were then added to 500 ml of lysis buffer in a 1000 ml beaker and mixed by stirring with a 2 ml pipette. The beaker containing the lysed cell suspension was placed on ice and sonicated for a total of 2.5 minutes (5–30 second bursts with 1 minute cooling between each) with a Braun sonicator, large probe, set at 100 watts power. Equal volumes of the solution were placed in Teflon SS34 centrifuge tubes and centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The pellets were resuspended in a total of 100 ml of sterile double distilled water by vortexing at high speed, and the centrifugation step repeated. Supernatants were discarded and the pellets combined in 20 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (Tris-buffered saline) and the suspension frozen overnight at –20° C.

The recombinant suspension was thawed at room temperature and added to 100 ml of 8 M Guanidine HCl (Sigma) in Tris-buffered saline and mixed vigorously. A magnetic stir bar was placed in the bottle and the solubilized sample was mixed at room temperature for 30 minutes. The solution was transferred to a 2000 ml Ehrlenmyer flask and 1200 ml of Tris-buffered saline was quickly added. This mixture was stirred at room temperature for an additional 2 hours. 500 ml aliquots were placed in dialysis bags (Spectrum, 63.7 mm diameter, 6,000–8,000 MW cutoff, #132670, from Fisher scientific) and these were placed in 4,000 ml beakers containing 3,500 ml of Tris-buffered saline+0.5 M Guanidine HCl. The beakers were placed in a 4° C. room on a magnetic stirrer overnight after which dialysis buffer was replaced with Tris-buffered saline+0.1 M Guanidine HCl and dialysis continued for 12 hours. The buffer was then replaced with Tris-buffered saline+0.05 M Guanidine HCl and dialysis continued overnight. The buffer was replaced with Tris-buffered saline (no guanidine), and dialysis continued for 12 hours. This was repeated three more times. The final solution was poured into a 2000 ml plastic roller bottle (Corning) and 13 ml of 100 mM PMSF (in ethanol) was added to inhibit protease activity. The solution was stored at –20° C. in 100 ml aliquots.

EXAMPLE 2

Measurement of IL2 Activity

Cell-free lysates were prepared by detergent lysis from *E. coli* carrying pAA356 as described above and an isogenic strain carrying the pGH433 vector without IL2-LKT. The IL2-LKT molecule was evident on polyacrylamide gel electrophoresis. IL2 activity was measured using an IL2-dependent T-cell line derived from Con-A-stimulated peripheral blood mononuclear cells. The recombinant lysates were added to IL2-dependent cells and proliferation was measured after 48 hours incubation at 37° C. The proliferative response to IL2 was compared to T lymphocytes cultured in medium alone or cells stimulated with recombinant human IL2 (specific activity=3.6×10^6 U/mg). Recombinant leukotoxin without IL2 was also included as a control. The results, shown in Table 1, confirm the IL2 activity of the fusion protein.

TABLE 1

IL2 Activity of IL2-LKT Fusion Product
Tested on an IL2-Dependent T-Cell Line*

	Counts Per Minute		
Sample	10^{-2}	10^{-3}	10^{-4}
Recombinant Leukotoxin	357	372	383
Vector Only (pGH433)	487	598	506
IL2-LKT (pAA356)	28,634	22,329	9,961

*Activity induced by recombinant human IL2 standards: 25 U/ml = 30,159 cpm; 12 U/ml = 23,666 cpm; 6 U/ml = 22,837 cpm; 3 U/ml = 15,828 cpm; 1.5 U/ml = 8,944 cpm; 0.6 U/ml = 3,233 cpm.

Thus, it is evident that the chimeric protein retains IL2 cell proliferative activity.

EXAMPLE 3

Serological Response to *P. haemolytica* LKT and the IL2-LKT Chimeric Molecule

To test whether the serological activity of the chimeric molecule differed from the serological activity of leukotoxin alone, the following experiment was done.

Calves (three per group) were immunized at time 0 with 100 μg of: (1) full-length recombinant *P. haemolytica* leukotoxin (LKT), (2) an equivalent molar ratio of the IL2-LKT chimeric protein, or (3) PBS. All of these were formulated in phosphate-buffered saline with Emulsigen as the adjuvant. Serological assessment of immune responsiveness to LKT or the chimera was carried out at -15, -7, -3 days and immediately prior to immunization on day 0, and daily for 20 days post-immunization. Serum antibody of the IgG class was assessed by enzyme-linked immunosorbent assay, using leukotoxin as the antigen.

As can be seen in FIGS. 4A–4C, the mean of individual serological titers in the nonimmunized group (FIG. 4A) remained at levels below 1/32 ($\log_2$ 5). One of the three calves in this group seroconverted to leukotoxin positive at day 20 because of natural infection with *P. haemolytica*. In the LKT-immunized group (4B), titers began to rise at day 6 after immunization, reaching a maximum (1/1024–1/8192; $\log_2$ 10–14) on day 8–10, where they remained for the duration of the experiment. In the chimera-immunized animals (4C), responses to LKT began to rise after day 4 postimmunization, reaching a maximum (1/1024–1/4096 $\log_2$ 10–12) on day 8 after immunization.

Thus, the serological activity of the chimeric molecule when compared to the activity of leukotoxin alone was not significantly different, both with respect to kinetics and magnitude. Serum antibody from one animal in the leukotoxin immunized group appeared to react with leukotoxin prior to immunization (with titers>1/128; $\log_2$ 7), and while it is unlikely that this animal suffered a *P. haemolytica* infection, serum antibodies against another bacterial toxin could be cross-reacting with leukotoxin. The conclusion from this experiment is that when IL2 is genetically chimerized to the leukotoxin molecule, it does not affect the ability of the LKT to induce a normal IgG antibody response when compared to the administration of recombinant leukotoxin alone.

EXAMPLE 4

Immunization of Calves with LKT and the IL2-LKT Chimeric Molecule

Calves were immunized at time 0 according to the protocols in Table 2. 117 micrograms of IL2-LKT were given (molar equivalent) and 100 micrograms of LKT given (molar equivalent).

TABLE 2

Calf Immunization Protocols

Antigen	Adjuvant	Number of Doses	Interval
LKT	Emulsigen-plus	5	12 H
IL2-LKT	Emulsigen-plus	5	12 H
IL2-LKT	Emulsigen-plus	1	
IL2-LKT	None	5	12 H

LKT refers to full-length leukotoxin.
IL2-LKT refers to LKT chimerized to bovine IL2.
In multiple-dose regimes, five doses were given at 12 h intervals over 2.5 days.

1. Precursor Frequency Analysis

The number of cells capable of responding to LKT following immunization was assessed using limiting dilution analysis (LDA). At the times indicated following immunization, T and B lymphocytes were isolated from peripheral blood by passing through Sephadex G-10 columns. Monocyte depletion was confirmed by flow cytometry. This cell population was diluted to various concentrations (10^5 to 10^2/ml) and added to 96-well plates in the presence of feeder cells (autologous 1500 rad irradiated PBMC) and antigen (LKT) at a previously determined optimal concentration (20 μg/ml). In some experiments, cells were stimulated with IL2-LKT (LKT356) or an equimolar concentration of IL2. Following incubation at 37° C. for 5 to 7 days, ^{3}H-thymidine was added to wells and cultures were harvested after an additional 24 hours incubation, counted and the percent negative cultures assessed following comparison with control cultures (i.e., cells cultured in the absence of antigen). Semi-Log_{10} plots were done of Log_{10} Percent negative cultures (Y) against number of cells plated (X). The number of cells responding at 37% negative cultures was calculated from an equation derived from the regression curve of Y versus X.

As can be seen in FIG. 5, the chimerization of LKT to IL2 does not affect the ability of PBMC to respond to the IL2 component of the molecule. Furthermore, precursor frequency analysis of cells responding to LKT or IL2-LKT yielded the following results: After immunization with LKT or IL2-LKT, with or without the adjuvant Emulsigen-plus, there was a dramatic increase in the number of cells responding to LKT. Following a single immunizing dose of IL2-LKT with Emulsigen-plus, there was no detectable increase in precursor frequency (Table 3).

2. Serology

Serum from the immunized calves was assessed for antibodies against LKT at the times indicated in Table 3. LKT antibodies were detected using standard ELISAs.

All animals showed an increased antibody titer against LKT following immunization. Increases were more marked in those animals given Emulsigen-plus in the formulation. Specifically, animals immunized with the chimera had a titer of 1/700 15 days after immunization, whereas when the same immunization was done with Emulsigen-plus, the titer was 1/35,000. Furthermore, even following one dose of IL2-LKT with Emulsigen-plus, the serological titer was 1/2500 (Table 3).

TABLE 3

Immunization[a]		Adjuvant[b]	Time (D)[c]	F[d]	Serology[e]
LKT	(M)	Emulsigen-plus	0	1:55657	1/150
			15	1:11087	1/6000
IL2-LKT	(M)	None	0	1:16728	1/200
			15	1:8976	1/700
IL2-LKT	(S)	Emulsigen-plus	0	1:50755	1/300
			15	1:117317	1/2500
IL2-LKT	(M)	None***	0	1:20728	1/1000
			15	1:16882	1/35000

[a]M: multiple dose regimen; S: single bonus dose.
[b]Adjuvant given with all doses. ***High values at time 0 may indicate a prior infection or x-reactivity.
[c]Time following first inoculation.
[d]Precursor frequency of B and T cells proliferating in response to LKT.
[e]Serology determined by ELISA using LKT as antigen.

Thus, this study demonstrated the ability of LKT and IL2-LKT formulations to elicit cellular and humoral immunity responses following single or multiple immunization. When Emulsigen-plus was used as an adjuvant, there was a high serological response. This was regardless of whether LKT or IL2-LKT was given as a single or multiple immunization regimen. The single dose inoculum gave a high humoral response (antibody titer) in the near absence of any detectable cellular response. The animal that elicited the highest cellular response after immunization was that which was given IL2-LKT alone. Therefore, IL2-LKT can elicit the

highest state of cellular reactivity. A higher humoral response can also be elicited by combining the chimeric protein with an adjuvant.

EXAMPLE 5

Construction of a γIFN-Leukotoxin Gene Fusion

To isolate the leukotoxin gene, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See Lo et al., *Infect. Immun.*, supra; *DNA Cloning*: Vols. I and II, supra; and T. MANIATIS et al., supra. A genomic library was constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See Lo et al., *Infect. Immun.*, supra. To confirm this, smaller fragments were recloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5' to 3' direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire leukotoxin gene sequence. The structure of this plasmid is shown in FIG. 1.

lktA, a MaeI restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the SmaI site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the ptac-based vector pGH432: laci digested with SmaI. One, pAA342, consisted of the 5'-AhaIII fragment of the lktA gene while the other, pAA345, contained the entire MaeI fragment described above. The clone pAA342 expressed a truncated leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3' end of the lktA gene (StyI BamHI fragment from pAA345) was ligated to StyI BamHI-digested pAA342, yielding the plasmid pAA352, which also expressed the truncated leukotoxin, termed LKT 352.

The coding sequence of the bovine γIFN gene from the plasmid pBOVIFNγ (CIBA-GEIGY, Basel, Switzerland), was cloned as a BalI/SspI fragment into pAA352 digested with BamHI and filled in with Klenow DNA Polymerase. The ligation mixture was transformed into *E. coli* strain JM105 and ampicillin-resistant transformants were selected. DNA from four transformants was analyzed by restriction endonuclease digestion and one plasmid, pAA497 (FIG. 6), was found to contain the interferon gene in the correct orientation. The nucleotide sequence and corresponding amino acid sequence of the fusion is shown in FIG. 7 (SEQ ID NO:3–4). The resulting fusion is a gene fusion of bovine γIFN to the 3'-end of the truncated lktA gene.

The recombinant fusion protein was purified as described in Example 1.3.

EXAMPLE 6

Measurement of γIFN Activity

Purified recombinant γIFN-LKT was prepared as described above. IFN activity was tested using three different assays:

1) Expression of MHC class II on monocytes and macrophages.
2) Inhibition of T cell proliferation.
3) Ability to inhibit viral replication.

1. Expression of MHC Class II on Monocytes and MacroPhages

Peripheral blood mononuclear cells (PBMC) were isolated from bovine venous blood and incubated at 37° C. for 18 hours with different concentrations of the γIFN-LKT chimera and molar equivalent amounts of recombinant bovine γIFN. Cells were then washed and resuspended in PBS-gelatin containing NaN_3. Cells were incubated with mouse monoclonal anti-MHC Class II antibody for 30 minutes followed by 30 minutes incubation with FITC labelled goat anti-mouse antibody. The percent positive and peak fluorescence was estimated using a Becton-Dickenson FACScan. Results are shown in Table 4. An elevation of peak fluorescence is an indication of interferon activity.

TABLE 4

	Peak Fluorescence		
Source Cells	Medium	γIFN	γIFN-LKT
Animal #1	114	153	140
Animal #2	120	139	140

2. Inhibition of T-Cell-Proliferation

Cells were incubated with Con-A in the presence of LKT, γIFN-LKT, or LKT+γIFN, and the proliferative response assessed following three days of incubation. Results are shown in Table 5. A decrease in this response is indicative of IFN activity.

TABLE 5

	Increased Proliferative Response		
Source Cells	Medium	γIFN-LKT	LKT + γIFN
Animal #1	++++	+/-	++
Animal #2	++++	-	++

3. Ability to Inhibit Viral Replication

The activity of γIFN-LKT was directly compared to the activity of equimolar quantities of γIFN in a standard VSV plaque inhibition assay using GBK cells as previously reported (Babiuk, L. A. and Rouse, B. T. (1976) *Infect. Immun.* 13:1567). Briefly, GBK cells growing in 96-well flat-bottom tissue culture plates (NUNC, Roskilde, DK)

were treated with two-fold dilutions of recombinant γIFN. After overnight incubation, the culture media was removed and 100 μl of fresh culture media containing 100 PFU of VSV was added to each well. After 2 hr of incubation, this virus inoculum was removed and the wells were overlayed with 200 μl of methyl cellulose/MEM. Culture plates were further incubated for 2 hr and stained with crystal violet. The antiviral titer was taken as the dilution of supernatants at which 50% of the cells were protected against VSV. The specific activity of the chimera was estimated as 78,000 units per mg protein.

EXAMPLE 7

Identification of Neutralizing Epitopes of Leukotoxin

As explained above, the *P. haemolytica* leukotoxin protein is a member of the RTX family of toxins and contains a series of repeated amino acid domains near the carboxy terminus. These domains are likely to be epitopes useful in the subject chimeric proteins. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn or Asp)-Asp, (SEQ ID NO:5) where X is Lys, Asp, Val or Asn. (Highlander et al. (1989) *DNA* 8:15–28; Welch, R. A. (1991) *Molec. Microbiol.* 5:521–528). However, other substitutions likely to render immunologically active peptides include substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

Based on this information, a synthetic peptide of the sequence GGNGDDFIDGGKGNDLLHGG (SEQ ID NO:6) was constructed by standard solid phase technology on an Applied Biosystems peptide synthesizer. Mice were immunized with authentic leukotoxins prepared from either *P. haemolytica*, or *Actinobacillus pleuropneumoniae* (serotypes 1 and 5) at 100 μg per dose with Freund's Complete Adjuvant (first vaccination) or Freund's Incomplete Adjuvant (all subsequent vaccinations). High titer serum samples from immunized mice were tested, in a standard ELISA, for the following: (1) their ability to react with recombinant and authentic *P. haemolytica* leukotoxin; (2) their ability to react with the toxin produced by *A. pleuropneumoniae*; and (3) their ability to react with the synthetic peptide described above. The results, summarized in Table 6, are expressed as the relative reactivity at a serum dilution of 1 in 100,000.

TABLE 6

Presence of Synthetic Peptide Epitopes in Toxins from *P. haemolytica* and *A. pleuropneumonia* serotypes 1 and 5

	Relative Serological Response To:		
Toxin Prepared From:	Synthetic Peptide	Actinobacillus Toxin	Pasteurella Toxin
A. pleuropneumoniae sero. 5	+++	++++	++
A. pleuropneumoniae	+	++++	+

TABLE 6-continued

Presence of Synthetic Peptide Epitopes in Toxins from *P. haemolytica* and *A. pleuropneumonia* serotypes 1 and 5

	Relative Serological Response To:		
Toxin Prepared From:	Synthetic Peptide	Actinobacillus Toxin	Pasteurella Toxin
sero. 1 *P. haemolytica*	+++	not determined	++++

This data indicated that animals vaccinated with either of the three leukotoxins developed antibodies which reacted with all toxins and a synthetic peptide based on a portion of the *P. haemolytica* toxin. Once an appropriate level of anti-peptide serum antibody was reached (ELISA titer of 100,000 or greater), spleen cells were fused with NS1 cells and monoclonal antibody-producing clones were isolated by standard techniques. Culture supernatants from these clones were tested for their ability to react with the synthetic peptide (above) and the respective toxins in an ELISA assay. The results for 2 clones are shown in Table 7.

TABLE 7

		Relative Reaction With:		
Clone	Immunogen	Pasteurella Toxin	Synthetic Peptide	Actinobacillus Toxin
ET122-6A4-3	Pasteurella toxin	++++	+++++	ND[1]
N37-3F9-6	Actinobacillus toxin	ND	++++	+++++

[1]Not determined

These results demonstrate that each of these monoclonal antibodies react with an epitope which is shared by the *P. haemolytica* and *A. pleuropneumoniae* toxins, and that this epitope is structurally similar to that of the synthetic peptide. This peptide is also structurally similar to a bovine rotavirus synthetic peptide of the sequence TMNGNEFQTGGIGNLPIRNWNAC, representing amino acids 40–60 of the VP6 protein. The monoclonal antibodies described above can therefore be used to determine the degree of their cross-reactivity with rotavirus proteins based on the epitope represented by the synthetic peptides. Furthermore, the immunologically active leukotoxin fragments might prove useful in immunizing against rotavirus.

These leukotoxin epitopes can be fused to cytokines such as IL2 and γIFN, or active fragments thereof, to form chimeric proteins for use in vaccine compositions.

Thus, chimeric proteins for use in stimulating immunity against pneumonia and other respiratory diseases have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 6

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3311 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..3294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GCT ACT GTT AAT AGA TCT GCA CCT ACT TCA AGC TCT ACG GGG AAC       48
Met Ala Thr Val Asn Arg Ser Ala Pro Thr Ser Ser Ser Thr Gly Asn
  1               5                  10                  15

ACA ATG AAA GAA GTG AAG TCA TTG CTG CTG GAT TTA CAG TTG CTT TTG       96
Thr Met Lys Glu Val Lys Ser Leu Leu Leu Asp Leu Gln Leu Leu Leu
            20                  25                  30

GAG AAA GTT AAA AAT CCT GAG AAC CTC AAG CTC TCC AGG ATG CAT ACA      144
Glu Lys Val Lys Asn Pro Glu Asn Leu Lys Leu Ser Arg Met His Thr
        35                  40                  45

TTT GAC TTT TAC GTG CCC AAG GTT AAC GCT ACA GAA TTG AAA CAT CTT      192
Phe Asp Phe Tyr Val Pro Lys Val Asn Ala Thr Glu Leu Lys His Leu
    50                  55                  60

AAG TGT TTA CTA GAA GAA CTC AAA CTT CTA GAG GAA GTG CTA AAT TTA      240
Lys Cys Leu Leu Glu Glu Leu Lys Leu Leu Glu Glu Val Leu Asn Leu
65                  70                  75                  80

GCT CCA AGC AAA AAC CTG AAC CCC AGA GAG ATC AAG GAT TCA ATG GAC      288
Ala Pro Ser Lys Asn Leu Asn Pro Arg Glu Ile Lys Asp Ser Met Asp
                85                  90                  95

AAT ATC AAG AGA ATC GTT TTG GAA CTA CAG GGA TCT GAA ACA AGA TTC      336
Asn Ile Lys Arg Ile Val Leu Glu Leu Gln Gly Ser Glu Thr Arg Phe
            100                 105                 110

ACA TGT GAA TAT GAT GAT GCA ACA GTA AAC GCT GTA GAA TTT CTG AAC      384
Thr Cys Glu Tyr Asp Asp Ala Thr Val Asn Ala Val Glu Phe Leu Asn
        115                 120                 125

AAA TGG ATT ACC TTT TGT CAA AGC ATC TAC TCA ACA ATG ACT GGG GAT      432
Lys Trp Ile Thr Phe Cys Gln Ser Ile Tyr Ser Thr Met Thr Gly Asp
    130                 135                 140

CTA AGC TTC CCT AGA CTT ACA ACC CTA TCA AAT GGG CTA AAA AAC ACT      480
Leu Ser Phe Pro Arg Leu Thr Thr Leu Ser Asn Gly Leu Lys Asn Thr
145                 150                 155                 160

TTA ACG GCA ACC AAA AGT GGC TTA CAT AAA GCC GGT CAA TCA TTA ACC      528
Leu Thr Ala Thr Lys Ser Gly Leu His Lys Ala Gly Gln Ser Leu Thr
                165                 170                 175

CAA GCC GGC AGT TCT TTA AAA ACT GGG GCA AAA AAA ATT ATC CTC TAT      576
Gln Ala Gly Ser Ser Leu Lys Thr Gly Ala Lys Lys Ile Ile Leu Tyr
            180                 185                 190

ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT AAT GGT TTA CAG      624
Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly Asn Gly Leu Gln
        195                 200                 205

GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG GTA CAA AGA GAA      672
Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu
    210                 215                 220
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GAA CGC AAT AAT ATT GCA ACA GCT CAA ACC AGT TTA GGC ACG ATT CAA      720
Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu Gly Thr Ile Gln
225                 230                 235                 240

ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA TCC GCT CCA CAA      768
Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln
                245                 250                 255

ATT GAT AAA TTG CTA CAG AAA ACT AAA GCA GGC CAA GCA TTA GGT TCT      816
Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser
            260                 265                 270

GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA ACT GTA TTA TCT      864
Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser
        275                 280                 285

GGC ATT CAA TCT ATT TTA GGC TCA GTA TTG GCT GGA ATG GAT TTA GAT      912
Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp
    290                 295                 300

GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC      960
Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly
305                 310                 315                 320

TTG GAG CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA     1008
Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys
                325                 330                 335

ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA     1056
Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu
            340                 345                 350

CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT ATC     1104
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile
        355                 360                 365

GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA GGG CTA     1152
Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser Gly Leu
    370                 375                 380

TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT AAA AAT GCT TCA     1200
Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser
385                 390                 395                 400

ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA AAC CAA GTT GTT     1248
Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala Asn Gln Val Val
                405                 410                 415

GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA GCC CAA CGT GTT     1296
Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala Gln Arg Val
            420                 425                 430

GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT GCT TTA ATT GCT TCT     1344
Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala Leu Ile Ala Ser
        435                 440                 445

ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC GGT ATT GCC GAT     1392
Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly Ile Ala Asp
    450                 455                 460

AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT TAT GCC GAA CGC TTT AAA     1440
Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala Glu Arg Phe Lys
465                 470                 475                 480

AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA TAT CAG CGG GGA     1488
Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly
                485                 490                 495

ACA GGG ACT ATT GAT GCA TCG GTT ACT GCA ATT AAT ACC GCA TTG GCC     1536
Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn Thr Ala Leu Ala
            500                 505                 510

GCT ATT GCT GGT GGT GTG TCT GCT GCT GCA GCC GGC TCG GTT ATT GCT     1584
Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala
        515                 520                 525

TCA CCG ATT GCC TTA TTA GTA TCT GGG ATT ACC GGT GTA ATT TCT ACG     1632
Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr
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    530                 535                 540

ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC GTT GCA AAT AAA     1680
Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His Val Ala Asn Lys
545                 550                 555                 560

ATT CAT AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT CAC GGT AAG AAC     1728
Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn
                565                 570                 575

TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG AAT TTA CAA GAT     1776
Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp
            580                 585                 590

AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA CAG GCA GAA CGT     1824
Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu Arg
        595                 600                 605

GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC ATT GGT GAT TTA     1872
Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly Asp Leu
    610                 615                 620

GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT GGT AAA GCC TAT     1920
Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr
625                 630                 635                 640

GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC GAT AAA TTA GTA     1968
Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala Asp Lys Leu Val
                645                 650                 655

CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT AAT TCG GGT AAA     2016
Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys
            660                 665                 670

GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG CCA TTA TTG ACG CCG GGA     2064
Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu Leu Thr Pro Gly
        675                 680                 685

ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT GAA TAT ATT ACC     2112
Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr Glu Tyr Ile Thr
    690                 695                 700

AAG CTC AAT ATT AAC CGT GTA GAT AGC TGG AAA ATT ACA GAT GGT GCA     2160
Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile Thr Asp Gly Ala
705                 710                 715                 720

GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG CGT ATT GGT ATT     2208
Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln Arg Ile Gly Ile
                725                 730                 735

GAA TTA GAC AAT GCT GGA AAT GTA ACT AAA ACC AAA GAA ACA AAA ATT     2256
Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys Glu Thr Lys Ile
            740                 745                 750

ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT GTT GGT TCT GGT     2304
Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly
        755                 760                 765

ACG ACG GAA ATT GAT GGC GGT GAA GGT TAC GAC CGA GTT CAC TAT AGC     2352
Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser
    770                 775                 780

CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC AAA GAG ACC GAG     2400
Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu
785                 790                 795                 800

CAA GGT AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC GGT AAA GCA CTA     2448
Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu
                805                 810                 815

CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC AAC CGT GAA GAA     2496
His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu
            820                 825                 830

AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT GCC GGT TAT TAC     2544
Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr Tyr
        835                 840                 845

ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC GGT ACA TCA CAT     2592
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Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr Ser His
    850                 855                 860

AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC TTT AAC GGT GGT     2640
Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe Asn Gly Gly
865                 870                 875                 880

GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT GAC CGC TTA TTT     2688
Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn Asp Arg Leu Phe
                885                 890                 895

GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT GGT GAT GAT TTT     2736
Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp Phe
            900                 905                 910

ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA CAC GGT GGC AAG GGC GAT     2784
Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly Gly Lys Gly Asp
        915                 920                 925

GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT ATT ATT ACC GAT     2832
Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp
    930                 935                 940

TCT GAC GGC AAT GAT AAA TTA TCA TTC TCT GAT TCG AAC TTA AAA GAT     2880
Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser Asn Leu Lys Asp
945                 950                 955                 960

TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC ACG AAT AGC AAA     2928
Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile Thr Asn Ser Lys
                965                 970                 975

AAA GAG AAA GTG ACC ATT CAA AAC TGG TTC CGA GAG GCT GAT TTT GCT     2976
Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala
            980                 985                 990

AAA GAA GTG CCT AAT TAT AAA GCA ACT AAA GAT GAG AAA ATC GAA GAA     3024
Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu
        995                 1000                1005

ATC ATC GGT CAA AAT GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT     3072
Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp
    1010                1015                1020

CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA     3120
Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys
1025                1030                1035                1040

GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC     3168
Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn
                1045                1050                1055

AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT     3216
Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn
            1060                1065                1070

GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT     3264
Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser
        1075                1080                1085

TTA TCT TCT CTT CAA TTT GCT AGG GGA TCC TAGCTAGCTA GCCATGG          3311
Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser
    1090                1095
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1098 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Thr Val Asn Arg Ser Ala Pro Thr Ser Ser Ser Thr Gly Asn
 1               5                  10                  15

Thr Met Lys Glu Val Lys Ser Leu Leu Leu Asp Leu Gln Leu Leu Leu
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            20                  25                  30

Glu Lys Val Lys Asn Pro Glu Asn Leu Lys Leu Ser Arg Met His Thr
         35                  40                  45

Phe Asp Phe Tyr Val Pro Lys Val Asn Ala Thr Glu Leu Lys His Leu
     50                  55                  60

Lys Cys Leu Leu Glu Glu Leu Lys Leu Leu Glu Glu Val Leu Asn Leu
 65                  70                  75                  80

Ala Pro Ser Lys Asn Leu Asn Pro Arg Glu Ile Lys Asp Ser Met Asp
                 85                  90                  95

Asn Ile Lys Arg Ile Val Leu Glu Leu Gln Gly Ser Glu Thr Arg Phe
            100                 105                 110

Thr Cys Glu Tyr Asp Asp Ala Thr Val Asn Ala Val Glu Phe Leu Asn
        115                 120                 125

Lys Trp Ile Thr Phe Cys Gln Ser Ile Tyr Ser Thr Met Thr Gly Asp
    130                 135                 140

Leu Ser Phe Pro Arg Leu Thr Thr Leu Ser Asn Gly Leu Lys Asn Thr
145                 150                 155                 160

Leu Thr Ala Thr Lys Ser Gly Leu His Lys Ala Gly Gln Ser Leu Thr
                165                 170                 175

Gln Ala Gly Ser Ser Leu Lys Thr Gly Ala Lys Lys Ile Ile Leu Tyr
            180                 185                 190

Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly Asn Gly Leu Gln
        195                 200                 205

Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu Val Gln Arg Glu
    210                 215                 220

Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu Gly Thr Ile Gln
225                 230                 235                 240

Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu Ser Ala Pro Gln
                245                 250                 255

Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln Ala Leu Gly Ser
            260                 265                 270

Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys Thr Val Leu Ser
        275                 280                 285

Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly Met Asp Leu Asp
    290                 295                 300

Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly
305                 310                 315                 320

Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys
                325                 330                 335

Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu
            340                 345                 350

Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn Ile
        355                 360                 365

Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser Gly Leu
    370                 375                 380

Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys Asn Ala Ser
385                 390                 395                 400

Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala Asn Gln Val Val
                405                 410                 415

Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu Ala Gln Arg Val
            420                 425                 430

Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala Leu Ile Ala Ser
        435                 440                 445
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Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala Gly Ile Ala Asp
    450                 455                 460

Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala Glu Arg Phe Lys
465                 470                 475                 480

Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu Tyr Gln Arg Gly
                485                 490                 495

Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn Thr Ala Leu Ala
            500                 505                 510

Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly Ser Val Ile Ala
        515                 520                 525

Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly Val Ile Ser Thr
    530                 535                 540

Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His Val Ala Asn Lys
545                 550                 555                 560

Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn His Gly Lys Asn
                565                 570                 575

Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala Asn Leu Gln Asp
            580                 585                 590

Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu Gln Ala Glu Arg
        595                 600                 605

Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn Ile Gly Asp Leu
    610                 615                 620

Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser Gly Lys Ala Tyr
625                 630                 635                 640

Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala Asp Lys Leu Val
                645                 650                 655

Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser Asn Ser Gly Lys
            660                 665                 670

Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu Leu Thr Pro Gly
        675                 680                 685

Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr Glu Tyr Ile Thr
    690                 695                 700

Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile Thr Asp Gly Ala
705                 710                 715                 720

Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln Arg Ile Gly Ile
                725                 730                 735

Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys Glu Thr Lys Ile
            740                 745                 750

Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe Val Gly Ser Gly
        755                 760                 765

Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg Val His Tyr Ser
    770                 775                 780

Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr Lys Glu Thr Glu
785                 790                 795                 800

Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr Gly Lys Ala Leu
                805                 810                 815

His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly Asn Arg Glu Glu
            820                 825                 830

Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His Ala Gly Tyr Tyr
        835                 840                 845

Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile Gly Thr Ser His
    850                 855                 860
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Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala Phe Asn Gly Gly
865                 870                 875                 880

Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn Asp Arg Leu Phe
                885                 890                 895

Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn Gly Asp Asp Phe
            900                 905                 910

Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly Gly Lys Gly Asp
        915                 920                 925

Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp Ile Ile Thr Asp
    930                 935                 940

Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser Asn Leu Lys Asp
945                 950                 955                 960

Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile Thr Asn Ser Lys
                965                 970                 975

Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu Ala Asp Phe Ala
            980                 985                 990

Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu Lys Ile Glu Glu
        995                 1000                1005

Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp
    1010                1015                1020

Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys
1025                1030                1035                1040

Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn
                1045                1050                1055

Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn
            1060                1065                1070

Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met Leu Asp Gln Ser
        1075                1080                1085

Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser
    1090                1095
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3229 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..3207

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ATG GCT ACT GTT ATA GAT CTA AGC TTC CCA AAA ACT GGG GCA AAA AAA      48
Met Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys Lys
  1               5                  10                  15

ATT ATC CTC TAT ATT CCC CAA AAT TAC CAA TAT GAT ACT GAA CAA GGT      96
Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly
            20                  25                  30

AAT GGT TTA CAG GAT TTA GTC AAA GCG GCC GAA GAG TTG GGG ATT GAG     144
Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu
        35                  40                  45

GTA CAA AGA GAA GAA CGC AAT AAT ATT GCA ACA GCT CAA ACC AGT TTA     192
Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu
    50                  55                  60

GGC ACG ATT CAA ACC GCT ATT GGC TTA ACT GAG CGT GGC ATT GTG TTA     240
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Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu
 65                  70                  75                  80

TCC GCT CCA CAA ATT GAT AAA TTG CTA CAG AAA ACT AAA GCA GGC CAA      288
Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln
                 85                  90                  95

GCA TTA GGT TCT GCC GAA AGC ATT GTA CAA AAT GCA AAT AAA GCC AAA      336
Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys
            100                 105                 110

ACT GTA TTA TCT GGC ATT CAA TCT ATT TTA GGC TCA GTA TTG GCT GGA      384
Thr Val Leu Ser Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly
        115                 120                 125

ATG GAT TTA GAT GAG GCC TTA CAG AAT AAC AGC AAC CAA CAT GCT CTT      432
Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu
    130                 135                 140

GCT AAA GCT GGC TTG GAG CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT      480
Ala Lys Ala Gly Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala
145                 150                 155                 160

AAT TCA GTA AAA ACA CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT      528
Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe
                165                 170                 175

GGT TCA AAA CTA CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA      576
Gly Ser Lys Leu Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys
            180                 185                 190

CTC AAA AAT ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT      624
Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val
        195                 200                 205

ATC TCA GGG CTA TTA TCG GGC GCA ACA GCT GCA CTT GTA CTT GCA GAT      672
Ile Ser Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp
    210                 215                 220

AAA AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA      720
Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala
225                 230                 235                 240

AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT TTA      768
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu
                245                 250                 255

GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG GCT GCT      816
Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala
            260                 265                 270

TTA ATT GCT TCT ACT GTT TCT CTT GCG ATT AGC CCA TTA GCA TTT GCC      864
Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala
        275                 280                 285

GGT ATT GCC GAT AAA TTT AAT CAT GCA AAA AGT TTA GAG AGT TAT GCC      912
Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala
    290                 295                 300

GAA CGC TTT AAA AAA TTA GGC TAT GAC GGA GAT AAT TTA TTA GCA GAA      960
Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu
305                 310                 315                 320

TAT CAG CGG GGA ACA GGG ACT ATT GAT GCA TCG GTT ACT GCA ATT AAT     1008
Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn
                325                 330                 335

ACC GCA TTG GCC GCT ATT GCT GGT GGT GTG TCT GCT GCT GCA GCC GGC     1056
Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly
            340                 345                 350

TCG GTT ATT GCT TCA CCG ATT GCC TTA TTA GTA TCT GGG ATT ACC GGT     1104
Ser Val Ile Ala Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly
        355                 360                 365

GTA ATT TCT ACG ATT CTG CAA TAT TCT AAA CAA GCA ATG TTT GAG CAC     1152
Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His
    370                 375                 380
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GTT GCA AAT AAA ATT CAT AAC AAA ATT GTA GAA TGG GAA AAA AAT AAT     1200
Val Ala Asn Lys Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn
385                 390                 395                 400

CAC GGT AAG AAC TAC TTT GAA AAT GGT TAC GAT GCC CGT TAT CTT GCG     1248
His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala
                405                 410                 415

AAT TTA CAA GAT AAT ATG AAA TTC TTA CTG AAC TTA AAC AAA GAG TTA     1296
Asn Leu Gln Asp Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu
            420                 425                 430

CAG GCA GAA CGT GTC ATC GCT ATT ACT CAG CAG CAA TGG GAT AAC AAC     1344
Gln Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn
        435                 440                 445

ATT GGT GAT TTA GCT GGT ATT AGC CGT TTA GGT GAA AAA GTC CTT AGT     1392
Ile Gly Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser
    450                 455                 460

GGT AAA GCC TAT GTG GAT GCG TTT GAA GAA GGC AAA CAC ATT AAA GCC     1440
Gly Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala
465                 470                 475                 480

GAT AAA TTA GTA CAG TTG GAT TCG GCA AAC GGT ATT ATT GAT GTG AGT     1488
Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser
                485                 490                 495

AAT TCG GGT AAA GCG AAA ACT CAG CAT ATC TTA TTC AGA ACG CCA TTA     1536
Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu
            500                 505                 510

TTG ACG CCG GGA ACA GAG CAT CGT GAA CGC GTA CAA ACA GGT AAA TAT     1584
Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr
        515                 520                 525

GAA TAT ATT ACC AAG CTC AAT ATT AAC CGT GTA GAT AGC TGG AAA ATT     1632
Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile
    530                 535                 540

ACA GAT GGT GCA GCA AGT TCT ACC TTT GAT TTA ACT AAC GTT GTT CAG     1680
Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln
545                 550                 555                 560

CGT ATT GGT ATT GAA TTA GAC AAT GCT GGA AAT GTA ACT AAA ACC AAA     1728
Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys
                565                 570                 575

GAA ACA AAA ATT ATT GCC AAA CTT GGT GAA GGT GAT GAC AAC GTA TTT     1776
Glu Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe
            580                 585                 590

GTT GGT TCT GGT ACG ACG GAA ATT GAT GGC GGT GAA GGT TAC GAC CGA     1824
Val Gly Ser Gly Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg
        595                 600                 605

GTT CAC TAT AGC CGT GGA AAC TAT GGT GCT TTA ACT ATT GAT GCA ACC     1872
Val His Tyr Ser Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr
    610                 615                 620

AAA GAG ACC GAG CAA GGT AGT TAT ACC GTA AAT CGT TTC GTA GAA ACC     1920
Lys Glu Thr Glu Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr
625                 630                 635                 640

GGT AAA GCA CTA CAC GAA GTG ACT TCA ACC CAT ACC GCA TTA GTG GGC     1968
Gly Lys Ala Leu His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly
                645                 650                 655

AAC CGT GAA GAA AAA ATA GAA TAT CGT CAT AGC AAT AAC CAG CAC CAT     2016
Asn Arg Glu Glu Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His
            660                 665                 670

GCC GGT TAT TAC ACC AAA GAT ACC TTG AAA GCT GTT GAA GAA ATT ATC     2064
Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile
        675                 680                 685

GGT ACA TCA CAT AAC GAT ATC TTT AAA GGT AGT AAG TTC AAT GAT GCC     2112
Gly Thr Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala
    690                 695                 700
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TTT AAC GGT GGT GAT GGT GTC GAT ACT ATT GAC GGT AAC GAC GGC AAT     2160
Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn
705                 710                 715                 720

GAC CGC TTA TTT GGT GGT AAA GGC GAT GAT ATT CTC GAT GGT GGA AAT     2208
Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn
                725                 730                 735

GGT GAT GAT TTT ATC GAT GGC GGT AAA GGC AAC GAC CTA TTA CAC GGT     2256
Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly
            740                 745                 750

GGC AAG GGC GAT GAT ATT TTC GTT CAC CGT AAA GGC GAT GGT AAT GAT     2304
Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp
        755                 760                 765

ATT ATT ACC GAT TCT GAC GGC AAT GAT AAA TTA TCA TTC TCT GAT TCG     2352
Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser
    770                 775                 780

AAC TTA AAA GAT TTA ACA TTT GAA AAA GTT AAA CAT AAT CTT GTC ATC     2400
Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile
785                 790                 795                 800

ACG AAT AGC AAA AAA GAG AAA GTG ACC ATT CAA AAC TGG TTC CGA GAG     2448
Thr Asn Ser Lys Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu
                805                 810                 815

GCT GAT TTT GCT AAA GAA GTG CCT AAT TAT AAA GCA ACT AAA GAT GAG     2496
Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu
            820                 825                 830

AAA ATC GAA GAA ATC ATC GGT CAA AAT GGC GAG CGG ATC ACC TCA AAG     2544
Lys Ile Glu Glu Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys
        835                 840                 845

CAA GTT GAT GAT CTT ATC GCA AAA GGT AAC GGC AAA ATT ACC CAA GAT     2592
Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp
    850                 855                 860

GAG CTA TCA AAA GTT GTT GAT AAC TAT GAA TTG CTC AAA CAT AGC AAA     2640
Glu Leu Ser Lys Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys
865                 870                 875                 880

AAT GTG ACA AAC AGC TTA GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT     2688
Asn Val Thr Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe
                885                 890                 895

ACC TCG TCT AAT GAT TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG     2736
Thr Ser Ser Asn Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met
            900                 905                 910

TTG GAT CAA AGT TTA TCT TCT CTT CAA TTT GCT AGG GGA TCC CAG GGC     2784
Leu Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser Gln Gly
        915                 920                 925

CAA TTT TTT AGA GAA ATA GAA AAC TTA AAG GAG TAT TTT AAT GCA AGT     2832
Gln Phe Phe Arg Glu Ile Glu Asn Leu Lys Glu Tyr Phe Asn Ala Ser
    930                 935                 940

AGC CCA GAT GTA GCT AAG GGT GGG CCT CTC TTC TCA GAA ATT TTG AAG     2880
Ser Pro Asp Val Ala Lys Gly Gly Pro Leu Phe Ser Glu Ile Leu Lys
945                 950                 955                 960

AAT TGG AAA GAT GAA AGT GAC AAA AAA ATT ATT CAG AGC CAA ATT GTC     2928
Asn Trp Lys Asp Glu Ser Asp Lys Lys Ile Ile Gln Ser Gln Ile Val
                965                 970                 975

TCC TTC TAC TTC AAA CTC TTT GAA AAC CTC AAA GAT AAC CAG GTC ATT     2976
Ser Phe Tyr Phe Lys Leu Phe Glu Asn Leu Lys Asp Asn Gln Val Ile
            980                 985                 990

CAA AGG AGC ATG GAT ATC ATC AAG CAA GAC ATG TTT CAG AAG TTC TTG     3024
Gln Arg Ser Met Asp Ile Ile Lys Gln Asp Met Phe Gln Lys Phe Leu
        995                 1000                1005

AAT GGC AGC TCT GAG AAA CTG GAG GAC TTC AAA AAG CTG ATT CAA ATT     3072
Asn Gly Ser Ser Glu Lys Leu Glu Asp Phe Lys Lys Leu Ile Gln Ile
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-continued

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    1010                1015                1020

CCG GTG GAT GAT CTG CAG ATC CAG CGC AAA GCC ATA AAT GAA CTC ATC     3120
Pro Val Asp Asp Leu Gln Ile Gln Arg Lys Ala Ile Asn Glu Leu Ile
1025                1030                1035                1040

AAA GTG ATG AAT GAC CTG TCA CCA AAA TCT AAC CTC AGA AAG CGG AAG     3168
Lys Val Met Asn Asp Leu Ser Pro Lys Ser Asn Leu Arg Lys Arg Lys
                1045                1050                1055

AGA AGT CAG AAT CTC TTT CGA GGC CGG AGA GCA TCA ACG TAATGGTCCT      3217
Arg Ser Gln Asn Leu Phe Arg Gly Arg Arg Ala Ser Thr
            1060                1065

CCTGCCTGCA AT                                                      3229
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1069 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Ala Thr Val Ile Asp Leu Ser Phe Pro Lys Thr Gly Ala Lys Lys
  1               5                  10                  15

Ile Ile Leu Tyr Ile Pro Gln Asn Tyr Gln Tyr Asp Thr Glu Gln Gly
            20                  25                  30

Asn Gly Leu Gln Asp Leu Val Lys Ala Ala Glu Glu Leu Gly Ile Glu
        35                  40                  45

Val Gln Arg Glu Glu Arg Asn Asn Ile Ala Thr Ala Gln Thr Ser Leu
    50                  55                  60

Gly Thr Ile Gln Thr Ala Ile Gly Leu Thr Glu Arg Gly Ile Val Leu
65                  70                  75                  80

Ser Ala Pro Gln Ile Asp Lys Leu Leu Gln Lys Thr Lys Ala Gly Gln
                85                  90                  95

Ala Leu Gly Ser Ala Glu Ser Ile Val Gln Asn Ala Asn Lys Ala Lys
            100                 105                 110

Thr Val Leu Ser Gly Ile Gln Ser Ile Leu Gly Ser Val Leu Ala Gly
        115                 120                 125

Met Asp Leu Asp Glu Ala Leu Gln Asn Asn Ser Asn Gln His Ala Leu
    130                 135                 140

Ala Lys Ala Gly Leu Glu Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala
145                 150                 155                 160

Asn Ser Val Lys Thr Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe
                165                 170                 175

Gly Ser Lys Leu Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys
            180                 185                 190

Leu Lys Asn Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val
        195                 200                 205

Ile Ser Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp
    210                 215                 220

Lys Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala
225                 230                 235                 240

Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile Leu
                245                 250                 255

Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val Ala Ala
            260                 265                 270
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-continued

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Leu Ile Ala Ser Thr Val Ser Leu Ala Ile Ser Pro Leu Ala Phe Ala
        275                 280                 285

Gly Ile Ala Asp Lys Phe Asn His Ala Lys Ser Leu Glu Ser Tyr Ala
    290                 295                 300

Glu Arg Phe Lys Lys Leu Gly Tyr Asp Gly Asp Asn Leu Leu Ala Glu
305                 310                 315                 320

Tyr Gln Arg Gly Thr Gly Thr Ile Asp Ala Ser Val Thr Ala Ile Asn
                325                 330                 335

Thr Ala Leu Ala Ala Ile Ala Gly Gly Val Ser Ala Ala Ala Ala Gly
            340                 345                 350

Ser Val Ile Ala Ser Pro Ile Ala Leu Leu Val Ser Gly Ile Thr Gly
        355                 360                 365

Val Ile Ser Thr Ile Leu Gln Tyr Ser Lys Gln Ala Met Phe Glu His
    370                 375                 380

Val Ala Asn Lys Ile His Asn Lys Ile Val Glu Trp Glu Lys Asn Asn
385                 390                 395                 400

His Gly Lys Asn Tyr Phe Glu Asn Gly Tyr Asp Ala Arg Tyr Leu Ala
                405                 410                 415

Asn Leu Gln Asp Asn Met Lys Phe Leu Leu Asn Leu Asn Lys Glu Leu
            420                 425                 430

Gln Ala Glu Arg Val Ile Ala Ile Thr Gln Gln Gln Trp Asp Asn Asn
        435                 440                 445

Ile Gly Asp Leu Ala Gly Ile Ser Arg Leu Gly Glu Lys Val Leu Ser
    450                 455                 460

Gly Lys Ala Tyr Val Asp Ala Phe Glu Glu Gly Lys His Ile Lys Ala
465                 470                 475                 480

Asp Lys Leu Val Gln Leu Asp Ser Ala Asn Gly Ile Ile Asp Val Ser
                485                 490                 495

Asn Ser Gly Lys Ala Lys Thr Gln His Ile Leu Phe Arg Thr Pro Leu
            500                 505                 510

Leu Thr Pro Gly Thr Glu His Arg Glu Arg Val Gln Thr Gly Lys Tyr
        515                 520                 525

Glu Tyr Ile Thr Lys Leu Asn Ile Asn Arg Val Asp Ser Trp Lys Ile
    530                 535                 540

Thr Asp Gly Ala Ala Ser Ser Thr Phe Asp Leu Thr Asn Val Val Gln
545                 550                 555                 560

Arg Ile Gly Ile Glu Leu Asp Asn Ala Gly Asn Val Thr Lys Thr Lys
                565                 570                 575

Glu Thr Lys Ile Ile Ala Lys Leu Gly Glu Gly Asp Asp Asn Val Phe
            580                 585                 590

Val Gly Ser Gly Thr Thr Glu Ile Asp Gly Gly Glu Gly Tyr Asp Arg
        595                 600                 605

Val His Tyr Ser Arg Gly Asn Tyr Gly Ala Leu Thr Ile Asp Ala Thr
    610                 615                 620

Lys Glu Thr Glu Gln Gly Ser Tyr Thr Val Asn Arg Phe Val Glu Thr
625                 630                 635                 640

Gly Lys Ala Leu His Glu Val Thr Ser Thr His Thr Ala Leu Val Gly
                645                 650                 655

Asn Arg Glu Glu Lys Ile Glu Tyr Arg His Ser Asn Asn Gln His His
            660                 665                 670

Ala Gly Tyr Tyr Thr Lys Asp Thr Leu Lys Ala Val Glu Glu Ile Ile
        675                 680                 685

Gly Thr Ser His Asn Asp Ile Phe Lys Gly Ser Lys Phe Asn Asp Ala
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-continued

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    690                 695                 700

Phe Asn Gly Gly Asp Gly Val Asp Thr Ile Asp Gly Asn Asp Gly Asn
705                 710                 715                 720

Asp Arg Leu Phe Gly Gly Lys Gly Asp Asp Ile Leu Asp Gly Gly Asn
                725                 730                 735

Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu Leu His Gly
            740                 745                 750

Gly Lys Gly Asp Asp Ile Phe Val His Arg Lys Gly Asp Gly Asn Asp
        755                 760                 765

Ile Ile Thr Asp Ser Asp Gly Asn Asp Lys Leu Ser Phe Ser Asp Ser
    770                 775                 780

Asn Leu Lys Asp Leu Thr Phe Glu Lys Val Lys His Asn Leu Val Ile
785                 790                 795                 800

Thr Asn Ser Lys Lys Glu Lys Val Thr Ile Gln Asn Trp Phe Arg Glu
                805                 810                 815

Ala Asp Phe Ala Lys Glu Val Pro Asn Tyr Lys Ala Thr Lys Asp Glu
            820                 825                 830

Lys Ile Glu Glu Ile Ile Gly Gln Asn Gly Glu Arg Ile Thr Ser Lys
        835                 840                 845

Gln Val Asp Asp Leu Ile Ala Lys Gly Asn Gly Lys Ile Thr Gln Asp
    850                 855                 860

Glu Leu Ser Lys Val Val Asp Asn Tyr Glu Leu Leu Lys His Ser Lys
865                 870                 875                 880

Asn Val Thr Asn Ser Leu Asp Lys Leu Ile Ser Ser Val Ser Ala Phe
                885                 890                 895

Thr Ser Ser Asn Asp Ser Arg Asn Val Leu Val Ala Pro Thr Ser Met
            900                 905                 910

Leu Asp Gln Ser Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser Gln Gly
        915                 920                 925

Gln Phe Phe Arg Glu Ile Glu Asn Leu Lys Glu Tyr Phe Asn Ala Ser
    930                 935                 940

Ser Pro Asp Val Ala Lys Gly Gly Pro Leu Phe Ser Glu Ile Leu Lys
945                 950                 955                 960

Asn Trp Lys Asp Glu Ser Asp Lys Lys Ile Ile Gln Ser Gln Ile Val
                965                 970                 975

Ser Phe Tyr Phe Lys Leu Phe Glu Asn Leu Lys Asp Asn Gln Val Ile
            980                 985                 990

Gln Arg Ser Met Asp Ile Ile Lys Gln Asp Met Phe Gln Lys Phe Leu
        995                 1000                1005

Asn Gly Ser Ser Glu Lys Leu Glu Asp Phe Lys Lys Leu Ile Gln Ile
    1010                1015                1020

Pro Val Asp Asp Leu Gln Ile Gln Arg Lys Ala Ile Asn Glu Leu Ile
1025                1030                1035                1040

Lys Val Met Asn Asp Leu Ser Pro Lys Ser Asn Leu Arg Lys Arg Lys
                1045                1050                1055

Arg Ser Gln Asn Leu Phe Arg Gly Arg Arg Ala Ser Thr
            1060                1065
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

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    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
         (A) NAME/KEY: Modified-site
         (B) LOCATION: 3
         (D) OTHER INFORMATION: /note= "X is Lys, Asp, Val or Asn."

    (ix) FEATURE:
         (A) NAME/KEY: Modified-site
         (B) LOCATION: 5
         (D) OTHER INFORMATION: /note= "X is Asn or Asp."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Gly Xaa Gly Xaa Asp
1               5


(2) INFORMATION FOR SEQ ID NO:6:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 20 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Gly Asn Gly Asp Asp Phe Ile Asp Gly Gly Lys Gly Asn Asp Leu
1               5                   10                  15

Leu His Gly Gly
            20
```

We claim:

1. A vaccine composition comprising an immunogenic chimeric protein that comprises gamma-interferon (γIFN), or an active fragment thereof, linked to at least one epitope of a leukotoxin derived from *Pasteurella haemolytica*, and a pharmaceutically acceptable vehicle.

2. The vaccine composition of claim 1 wherein said chimeric protein is linked to carrier.

3. A method of preventing or ameliorating respiratory disease comprising administering to a subject ruminant an effective amount of a vaccine composition according to claim 1.

4. The vaccine composition of claim 1, wherein said leukotoxin is full-length *P. haemolytica* leukotoxin.

5. The vaccine composition of claim 1, wherein said leukotoxin is a truncated leukotoxin that lacks cytotoxic activity.

6. The vaccine composition of claim 5, wherein said truncated leukotoxin is LKT 352.

7. The vaccine composition of claim 1, wherein said γIFN is bovine γIFN, or an active fragment thereof.

8. The vaccine composition of claim 7, wherein said chimeric protein comprises an amino acid sequence (a) encoded by a polynucleotide that encodes the LKT-γIFN amino acid sequence of SEQ ID NO:4, or (b) encoded by a polynucleotide that hybridizes to the polynucleotide of (a) under stringent hybridization conditions.

9. The vaccine composition of claim 1, further comprising an adjuvant.

10. The vaccine composition of claim 8, further comprising an adjuvant.

11. A method of preventing or ameliorating respiratory disease comprising administering to a subject ruminant an effective amount of a vaccine composition according to claim 8.

12. The vaccine composition of claim 8, wherein said chimeric protein comprises the LKT-γIFN amino acid sequence of SEQ ID NO:4.

13. A method of preventing or ameliorating respiratory disease comprising administering to a subject ruminant an effective amount of a vaccine composition according to claim 12.

* * * * *

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ACLM/"therapeutic benefit": 48 patents.
Hits 1 through 48 out of 48

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Refine Search ACLM/"therapeutic benefit"

	PAT. NO.	Title
1	6,096,885	Oxoazepine derivatives
2	6,090,810	Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
3	6,043,252	Carboline derivatives
4	6,032,677	Method and apparatus for stimulating the healing of medical implants
5	6,008,204	Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
6	6,001,847	Chemical compounds
7	5,985,326	Method of producing a solid dispersion of a poorly water soluble drug
8	5,981,527	Cyclic GMP-specific phosphodiesterase inhibitors
9	5,977,136	Tetrahydroquinolines as NMDA antagonists
10	5,958,954	Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
11	5,952,345	Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
12	5,891,473	Granular Compositions
13	5,889,182	Oxoazepine derivatives
14	5,885,829	Engineering oral tissues
15	5,877,207	Synthesis and use of retinoid compounds having negative hormone and/or antagonist activities
16	5,863,931	Amidino derivatives and their use as nitric oxide synthase inhibitors
17	5,855,616	Bioartificial pancreas
18	5,760,059	Indole derivatives
19	5,747,544	Method of using pure 3R-3'R stereoisomer of zeaxanthin to treat or prevent retinal degeneration in humans
20	5,739,129	CCK or gastrin modulating 5-heterocyclic-1, 5 benzodiazepines
21	5,733,867	1, 5-benzodiazepine derivatives

22 5,726,179 Muscarinic agonists
23 5,716,953 1,5-benzodiazepine derivatives
24 5,686,461 Indole derivatives
25 5,646,140 1,5-benzodiazepine derivatives having CCK antagonistic or agonistic activity
26 5,641,775 3-phenylureido-1,5-benzodiazepine-diones useful as gastrin or antagonists
27 5,620,438 Method and apparatus for treating vascular tissue following angioplasty to minimize restenosis
28 5,618,818 Muscarinic agonist compounds
29 5,605,911 Use of alpha-2 adrenergic drugs to prevent adverse effects of NMDA receptor hypofunction (NRH)
30 5,580,895 1,5-benzodiazepine derivatives
31 5,569,654 Benzodiazepinones
32 5,486,514 Carbamate derivatives
33 5,449,376 System and method for producing highly amplified radio signals for feedback into the human body
34 5,425,764 Bioartificial pancreas
35 5,403,845 Muscarinic agonists
36 5,374,649 Indole derivatives and pharmaceutical use thereof
37 5,374,648 Indole derivatives and pharmaceutical use thereof
38 5,175,166 Muscarinic agonists
39 4,895,846 Pharmaceutically useful dihydropyridinyldicarboxylate amides and esters incorporating arylpiperazinylalkyl moieties
40 4,755,512 Pharmaceutically useful dihydropyridinyldicarboxylate amides and esters incorporating arylpiperazinylalkyl moities
41 4,743,612 Prodrug derivatives of the cardiotonic agent 4-ethyl-1,3-dihydro-5-(4-(2-methyl-1H-imidazol-1-yl)benzoyl)-2H-imidazol -2-one, composition containing them, and method of using them to treat cardiac failure
42 4,737,511 Cardiotonic imidazolylphenylpyrrol-2-ones
43 4,638,806 Rectal hemorrhoid therapeutic apparatus
44 4,556,665 Cardiotonic 1,3-dihydro-4-[[(imidazol-1-yl)aryl]carbonyl]imidazol-2-ones
45 4,517,310 N-[2-hydroxy-2-(3-hydroxyphenyl)ethyl]-1H-benzimidazole-1-butanamine and use thereof as a cardiotonic agent
46 4,414,213 Dihydropyridyl cyclic imidate esters and their pharmaceutical use
47 4,363,808 N-(3-Phenoxy-2-hydroxypropyl)benzimidazole-1-alkanamines
48 4,234,595 3-Indolyl-tertiary butylaminopropanols

US006090810A

United States Patent [19]

Klein et al.

[11] Patent Number: 6,090,810

[45] Date of Patent: *Jul. 18, 2000

[54] **SYNTHESIS AND USE OF RETINOID COMPOUNDS HAVING NEGATIVE HORMONE AND/OR ANTAGONIST ACTIVITIES**

[75] Inventors: **Elliott S. Klein**, Marina del Rey; **Alan T. Johnson**, Rancho Santa Margarita; **Andrew M. Standeven**, Corona del Mar; **Richard L. Beard**, Newport Beach; **Samuel J. Gillett**, Albany; **Tien T. Duong**, Irvine; **Sunil Nagpal**, Lake Forest; **Vidyasagar Vuligonda**, Irvine; **Min Teng**, Aliso Viejo; **Roshantha A. Chandraratna**, Mission Viejo, all of Calif.

[73] Assignee: **Allergan Sales, Inc.**, Irvine, Calif.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **09/222,984**

[22] Filed: **Dec. 30, 1998**

Related U.S. Application Data

[62] Division of application No. 08/871,093, Jun. 9, 1997, Pat. No. 5,952,345, which is a division of application No. 08/613,863, Mar. 11, 1996, Pat. No. 5,776,699.

[60] Provisional application No. 60/020,501, Oct. 13, 1995, provisional application No. 60/019,015, Sep. 1, 1995, and provisional application No. 60/064,853, Sep. 1, 1995.

[51] **Int. Cl.**[7] **C07D 409/14**; C07D 401/12

[52] **U.S. Cl.** **514/253**; 549/404; 549/405; 549/406; 549/410

[58] **Field of Search** 514/253, 254, 514/269, 274, 275, 314, 333, 336, 340, 341, 432, 456; 544/238, 315, 319, 328, 331, 333, 405; 546/167, 169, 170, 171, 173, 174, 176, 256, 271.1, 269.7, 280.1, 282.7; 549/23, 404, 405, 406, 410

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Primary Examiner—Richard L. Raymond
Assistant Examiner—Deepak R. Rao
Attorney, Agent, or Firm—Gabor L. Szekeres; Robert J. Baran; Martin A. Voet

[57] **ABSTRACT**

Aryl-substituted and aryl and (3-oxo-1-propenly)-substituted benzopyran, benzothiopyran, 1,2-dihydroquinoline, and 5,6-dihydronaphthalene derivatives have retinoid negative hormone and/or antagonist-like biological activities. The invented RAR antagonists can be administered to mammals, including humans, for the purpose of preventing or diminishing action of RAR agonists on the bound receptor sites. Specifically, the RAR agonists are administered or coadministered with retinoid drugs to prevent or ameliorate toxicity or side effects caused by retinoids or vitamin A or vitamin A precursors. The retinoid negative hormones can be used to potentiate the activities of other retinoids and nuclear receptor agonists. For example, the retinoid negative hormone called AGN 193109 effectively increased the effectiveness of other retinoids and steroid hormones in in vitro transactivation assays. Additionally, transactivation assays can be used to identify compounds having negative hormone activity. These assays are based on the ability of negative hormones to down-regulate the activity of chimeric retinoid receptors engineered to possess a constitutive transcription activator domain.

38 Claims, 15 Drawing Sheets

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5,354,752	10/1994	Chandraratna	514/252
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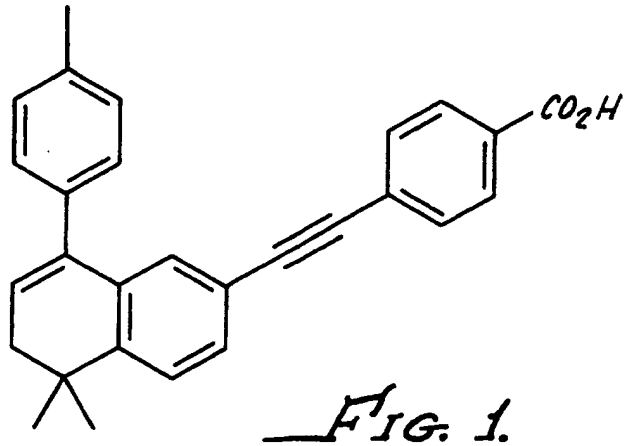


FIG. 1.

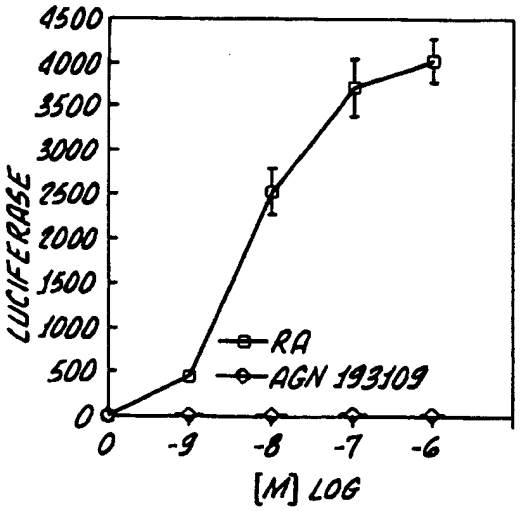
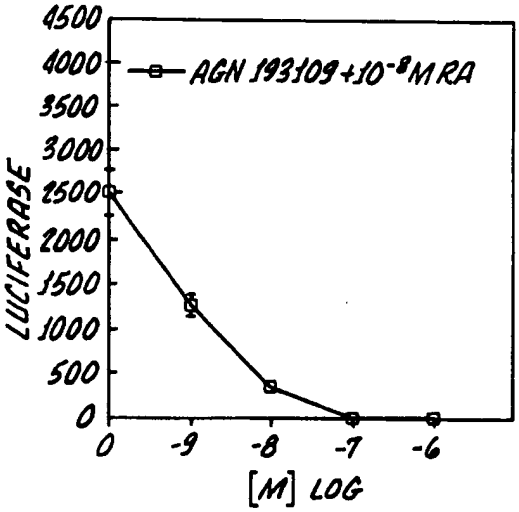


FIG. 2A.



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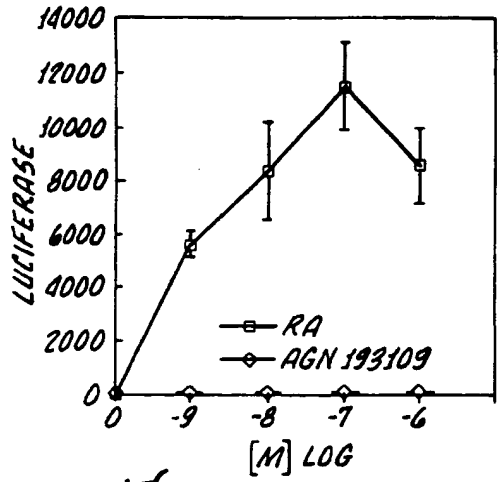

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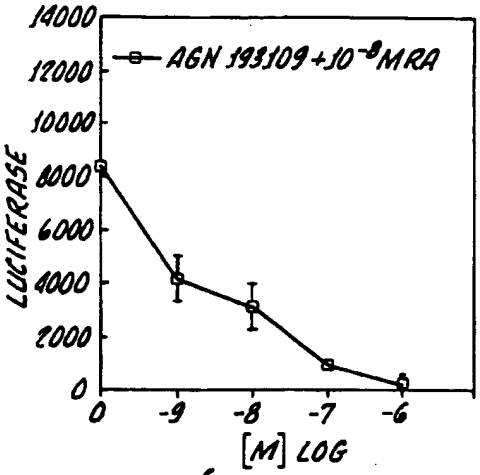
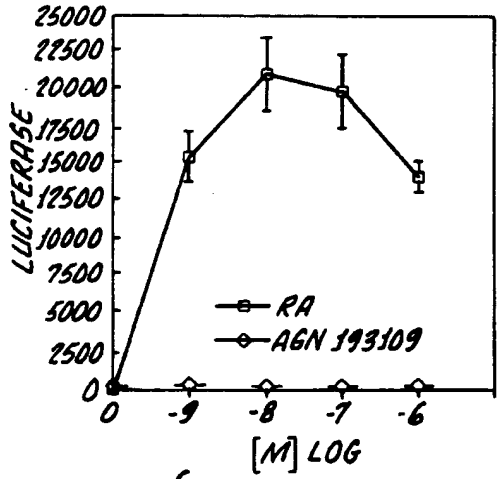


FIG. 2D.



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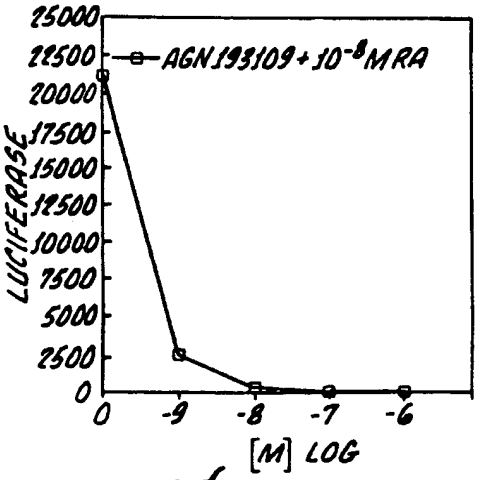

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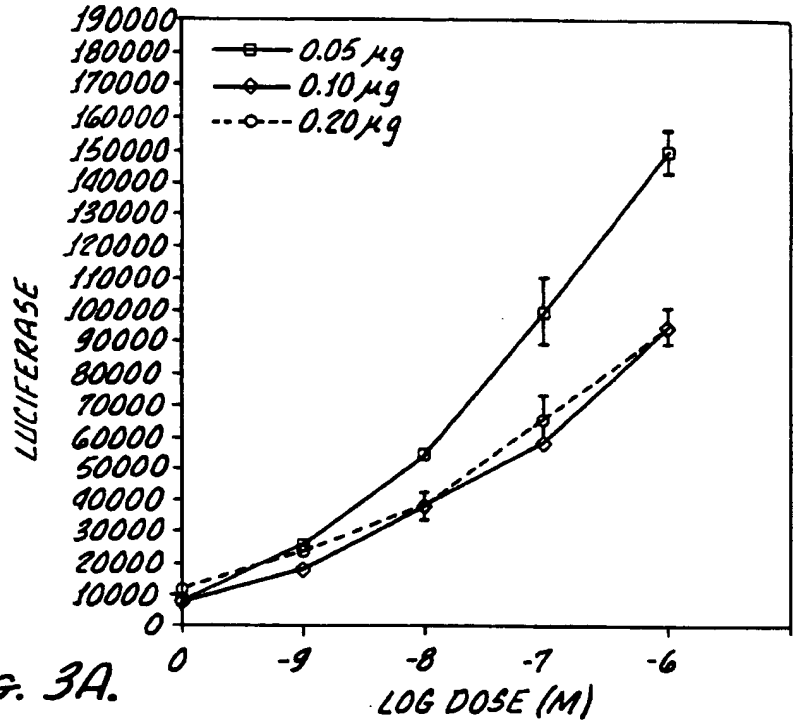
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160000
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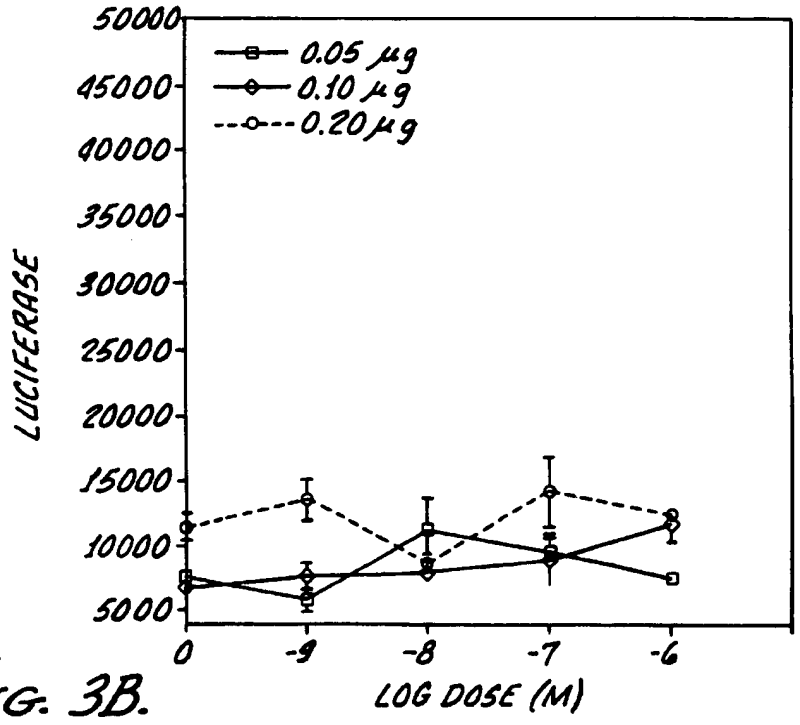
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
LUCIFERASE
0.05 µg
0.10 µg
0.20 µg
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 3A.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40000
35000
30000
25000
20000
15000
10000
5000
LUCIFERASE
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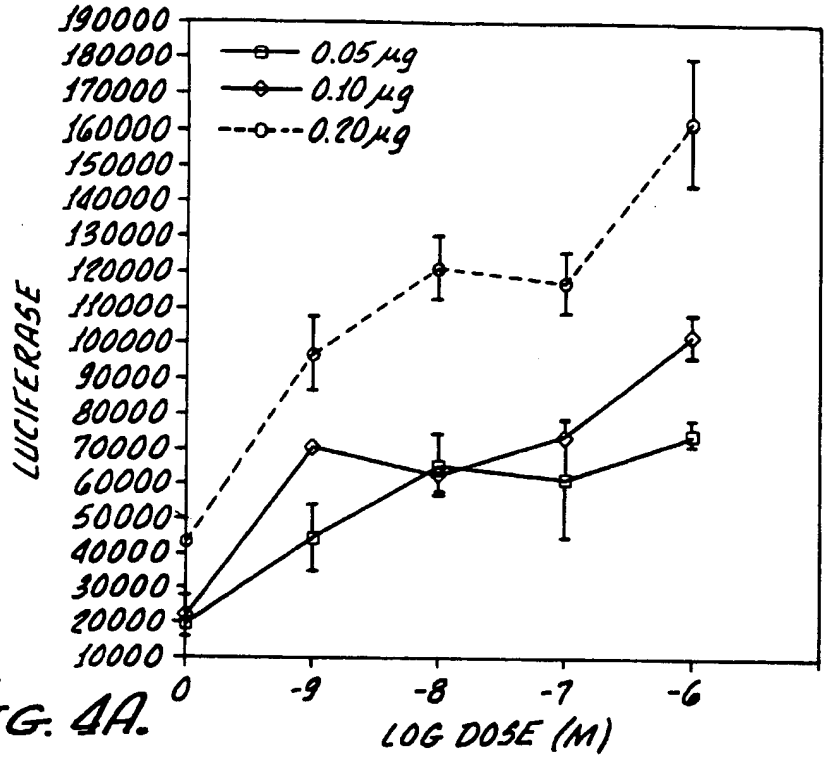
0.10 µg
0.20 µg
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 3B.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180000
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160000
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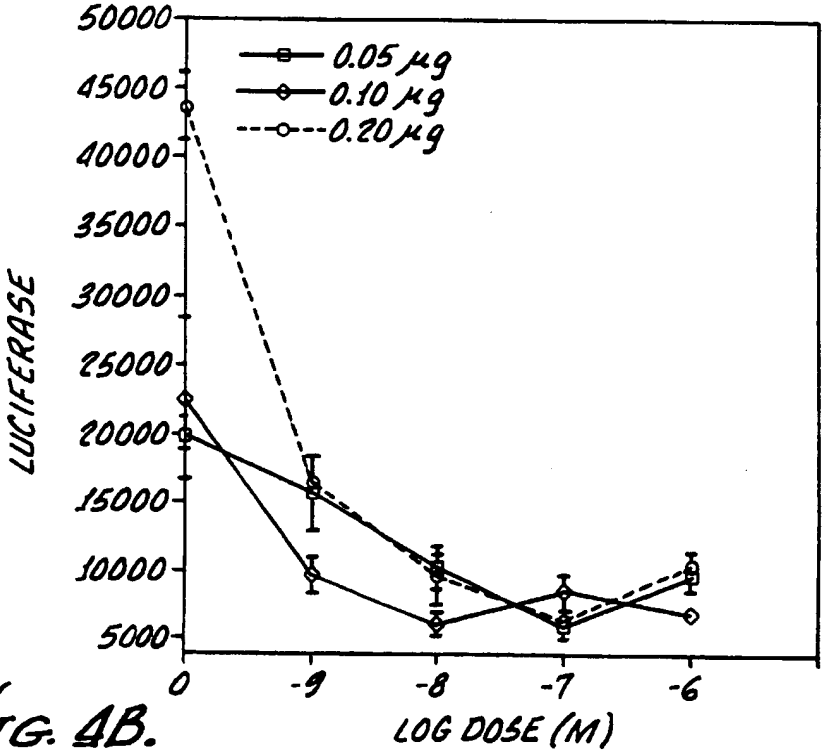
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
LUCIFERASE
0.05 µg
0.10 µg
0.20 µg
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 4A.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45000
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35000
30000
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15000
10000
5000
LUCIFERASE
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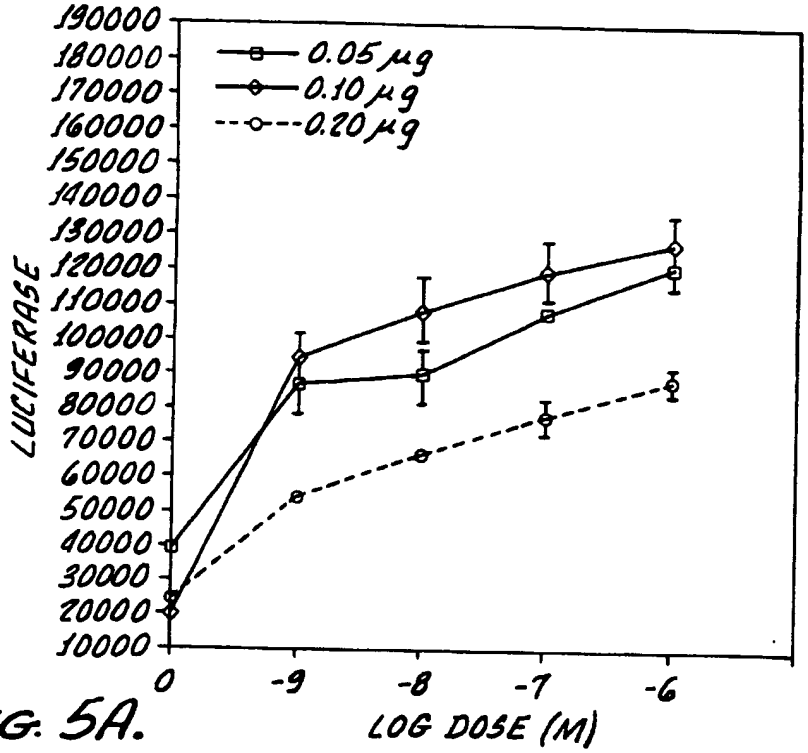
0.10 µg
0.20 µg
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 4B.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100000
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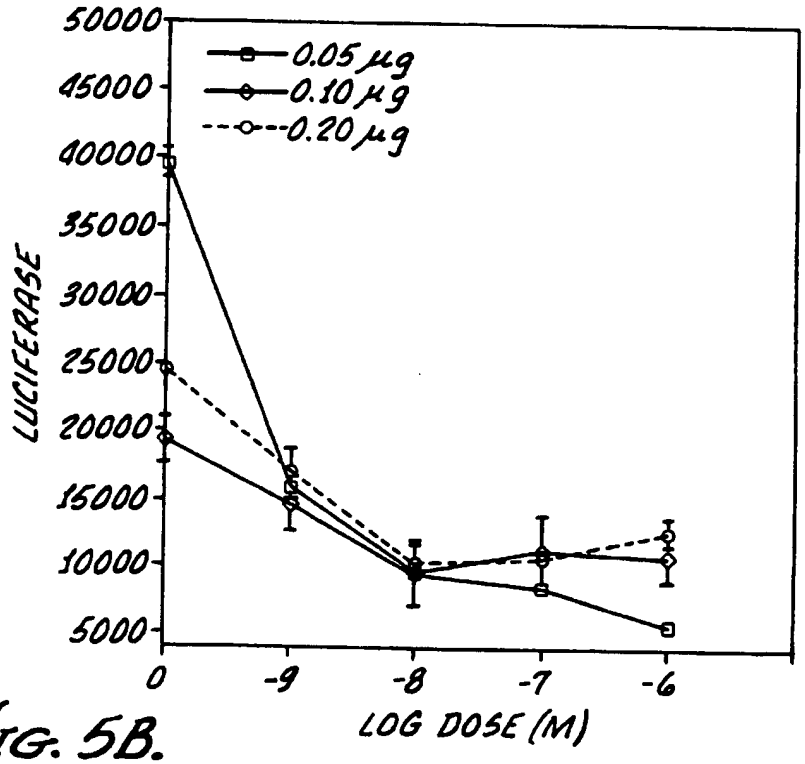
80000
70000
60000
50000
40000
30000
20000
10000
LUCIFERASE
0.05 µg
0.10 µg
0.20 µg
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 5A.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0.10 µg
0.20 µg
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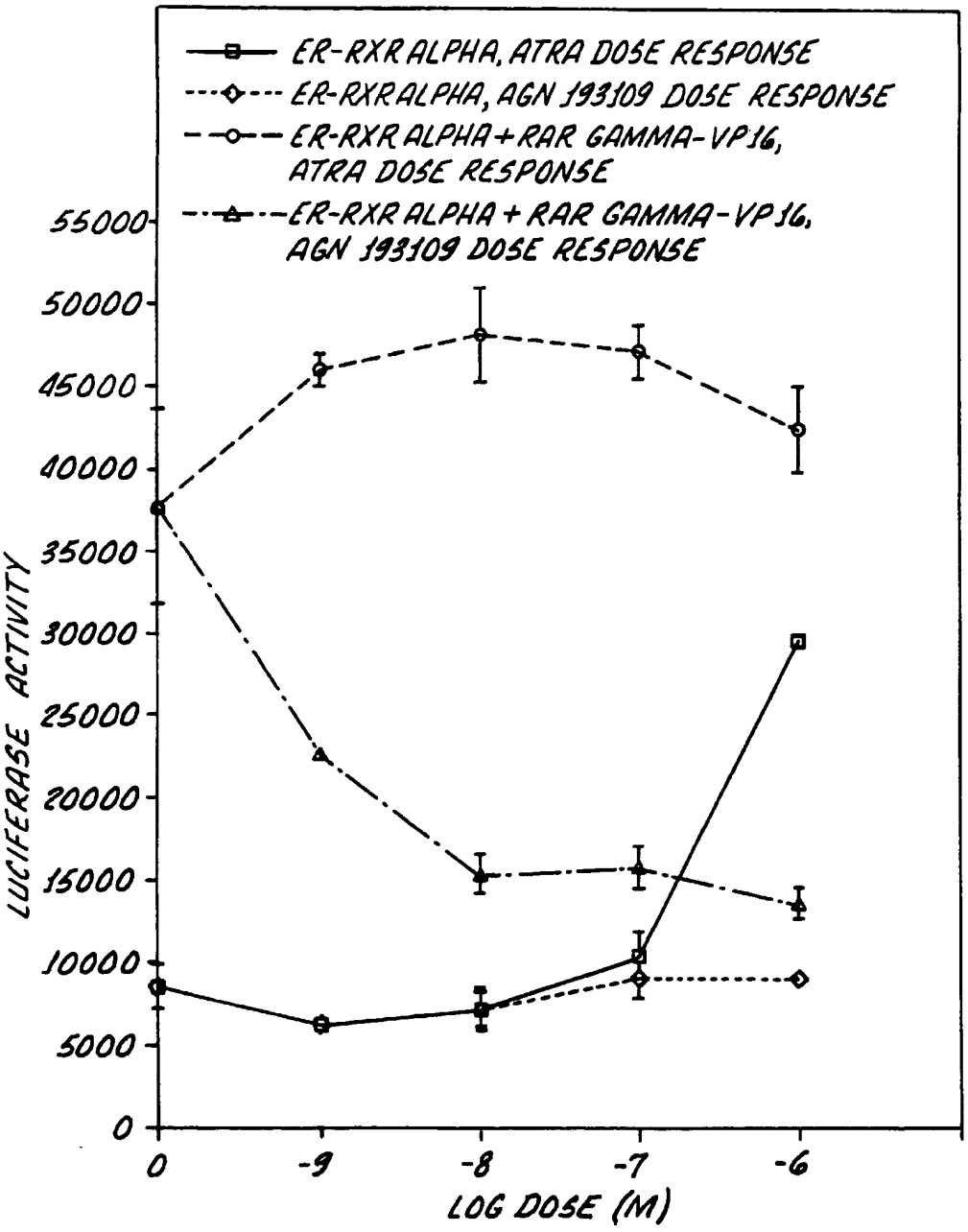
-8
-7
-6
LOG DOSE (M)

FIG. 5B.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ER-RXR ALPHA, AGN 193109 DOSE RESPONSE
ER-RXR ALPHA + RAR GAMMA-VP16, ATRA DOSE RESPONSE
ER-RXR ALPHA + RAR GAMMA-VP16, AGN 193109 DOSE RESPONSE
LUCIFERASE ACTIVITY
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
-9
-8
-7
-6
LOG DOSE (M)

FIG. 6.

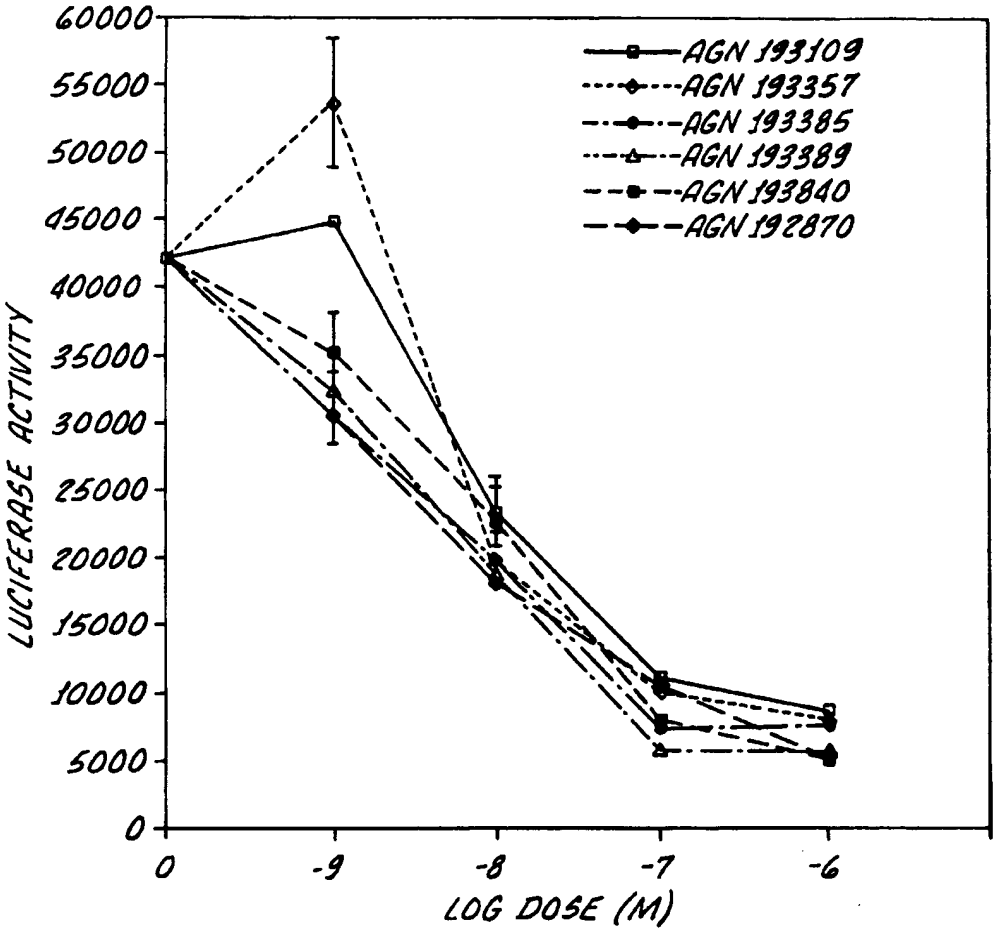

60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
LUCIFERASE ACTIVITY
0
-9
-8
-7
-6
LOG DOSE (M)
AGN 193109
AGN 193357
AGN 193385
AGN 193389
AGN 193840
AGN 192870

FIG. 7.

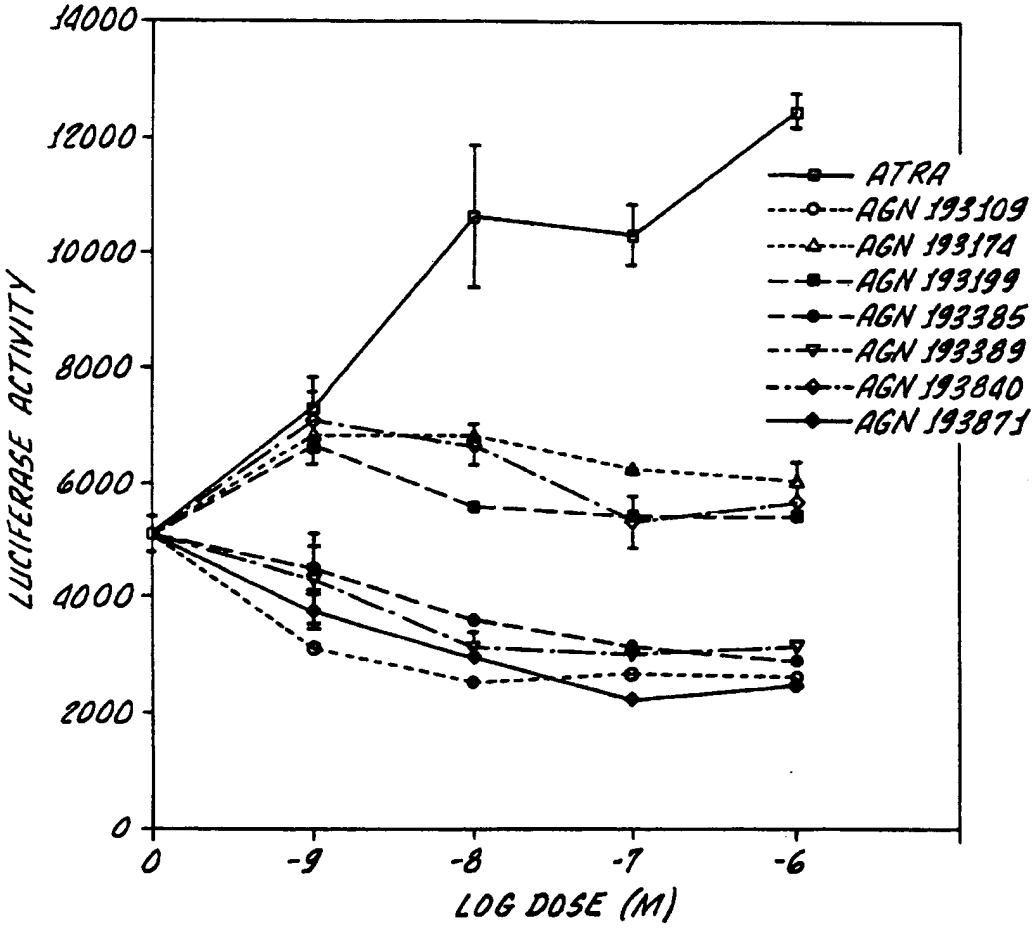

14000
12000
10000
8000
6000
4000
2000
0
LUCIFERASE ACTIVITY
0
-9
-8
-7
-6
LOG DOSE (M)
ATRA
AGN 193109
AGN 193174
AGN 193199
AGN 193385
AGN 193389
AGN 193840
AGN 193871

FIG. 8.

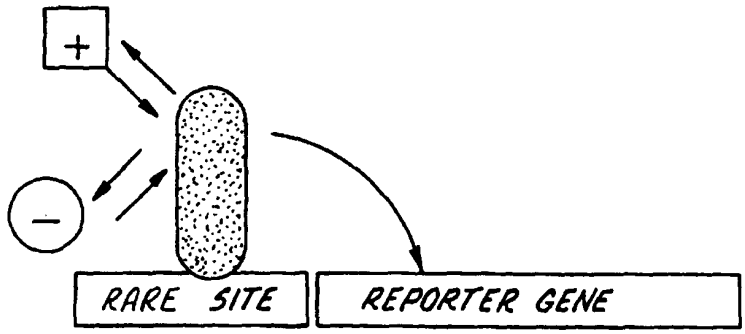


FIG. 9A.
NO LIGAND

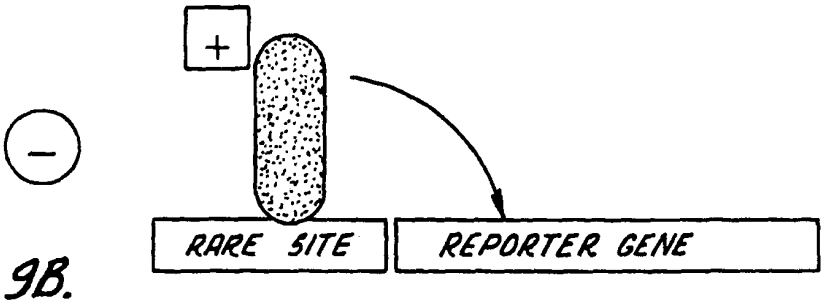


FIG. 9B.
ADDITION OF AGONIST

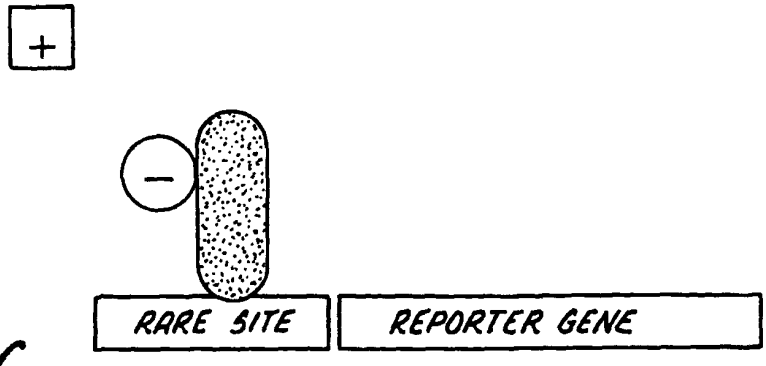


FIG. 9C.
ADDITION OF AGN 193109

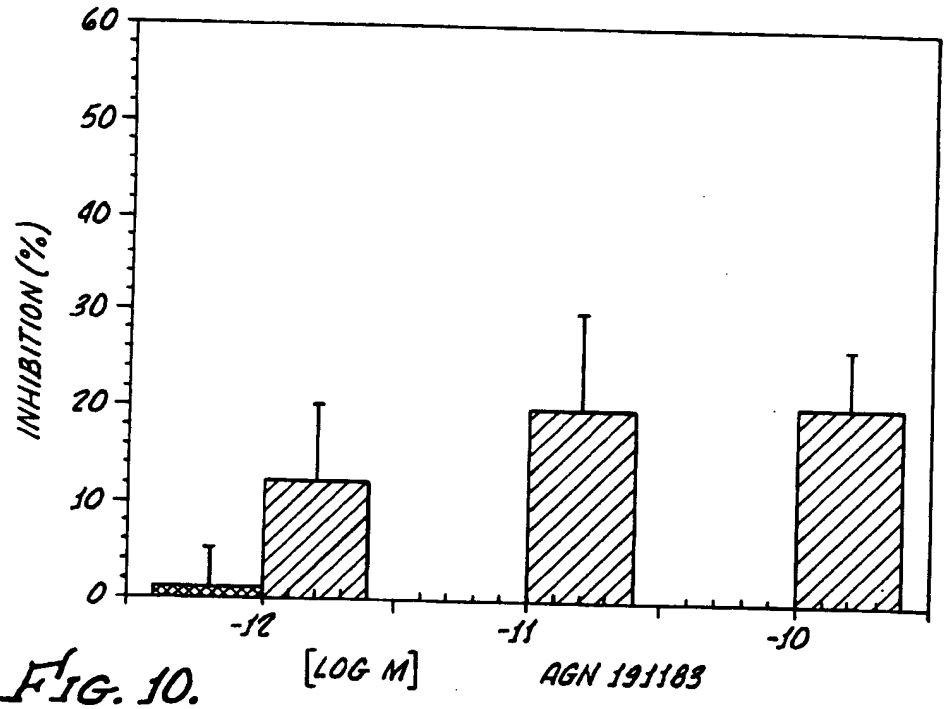

60
50
40
30
20
10
0
INHIBITION (%)
-12
-11
-10
[LOG M]
AGN 191183

FIG. 10.

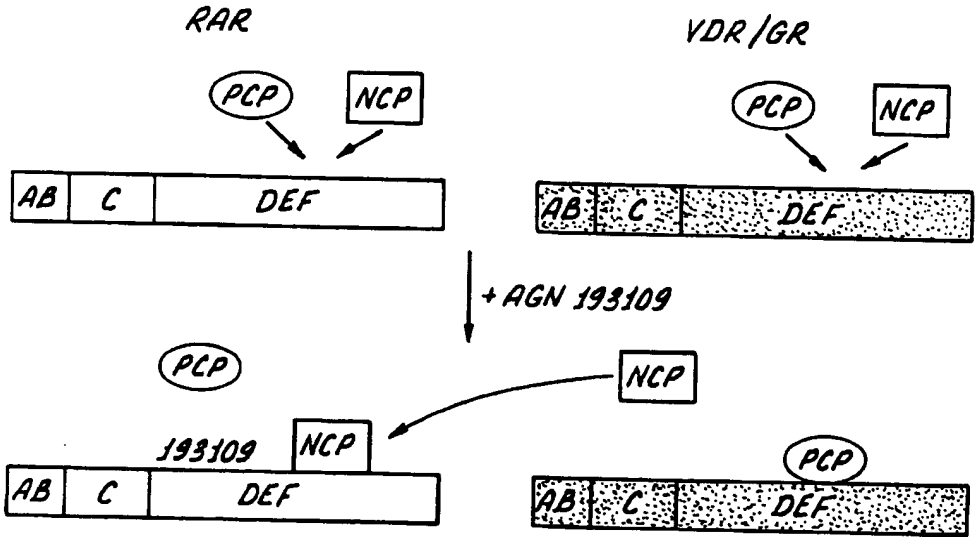

RAR
VDR/GR
PCP
NCP
AB
C
DEF
+ AGN 193109
193109

FIG. 11.

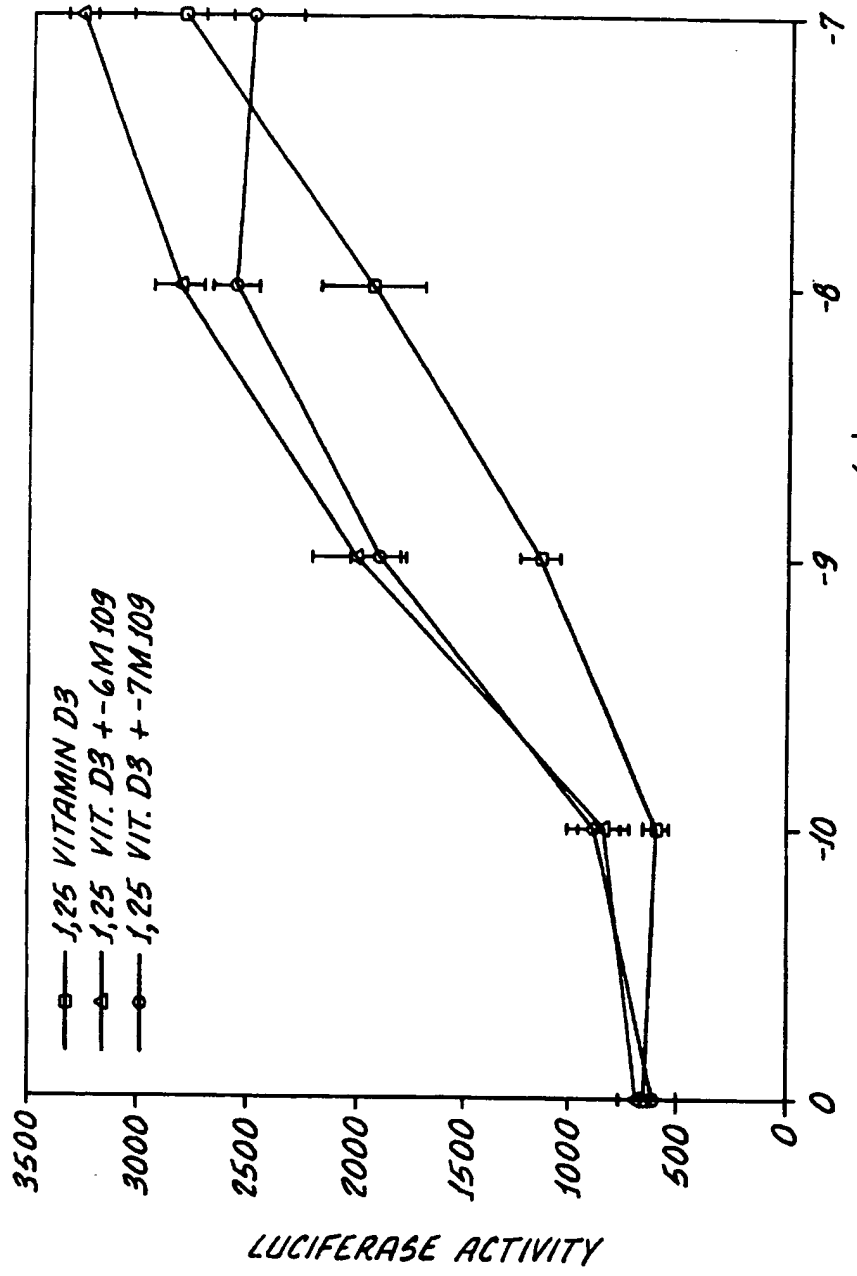

1,25 VITAMIN D3
1,25 VIT. D3 +-6M 109
1,25 VIT. D3 +-7M 109
LUCIFERASE ACTIVITY
3500
3000
2500
2000
1500
1000
500
0
-7
-8
-9
-10
0
LOG DOSE (M)

FIG. 12.

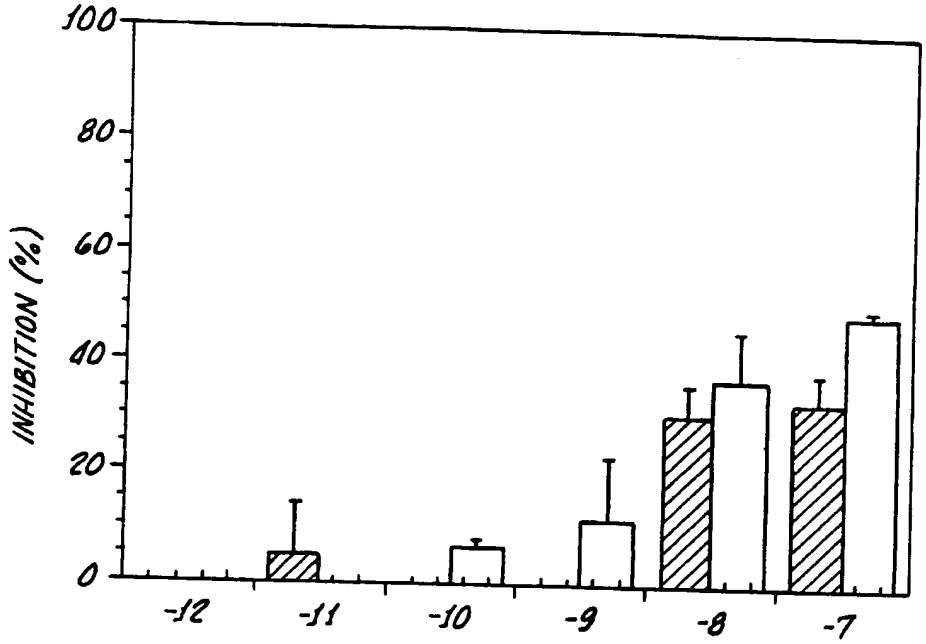

100
80
60
40
20
0
INHIBITION (%)
-12
-11
-10
-9
-8
-7
1,25 DIHYDROXY VITAMIN D3 [LOG M]

FIG. 13.

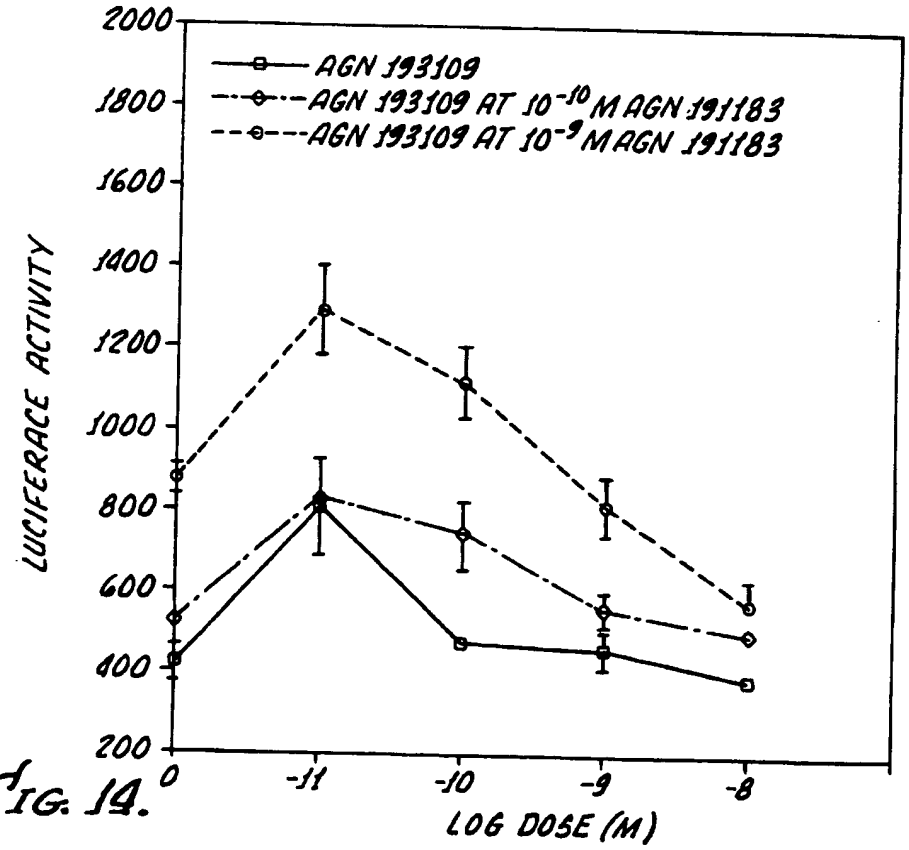

2000
1800
1600
1400
1200
1000
800
600
400
200
LUCIFERACE ACTIVITY
AGN 193109
AGN 193109 AT 10-10 M AGN 191183
AGN 193109 AT 10-9 M AGN 191183
0
-11
-10
-9
-8
LOG DOSE (M)

FIG. 14.

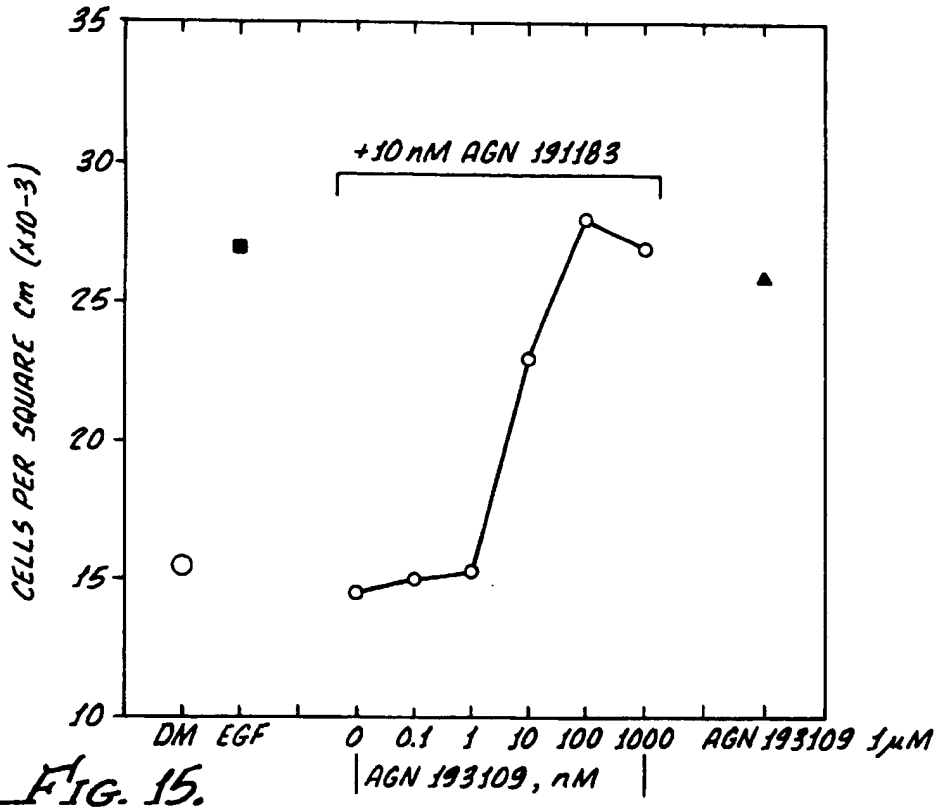

35
30
25
20
15
10
CELLS PER SQUARE Cm (x10-3)
+10 nM AGN 191183
DM EGF 0 0.1 1 10 100 1000 AGN 193109 1μM
AGN 193109, nM

FIG. 15.

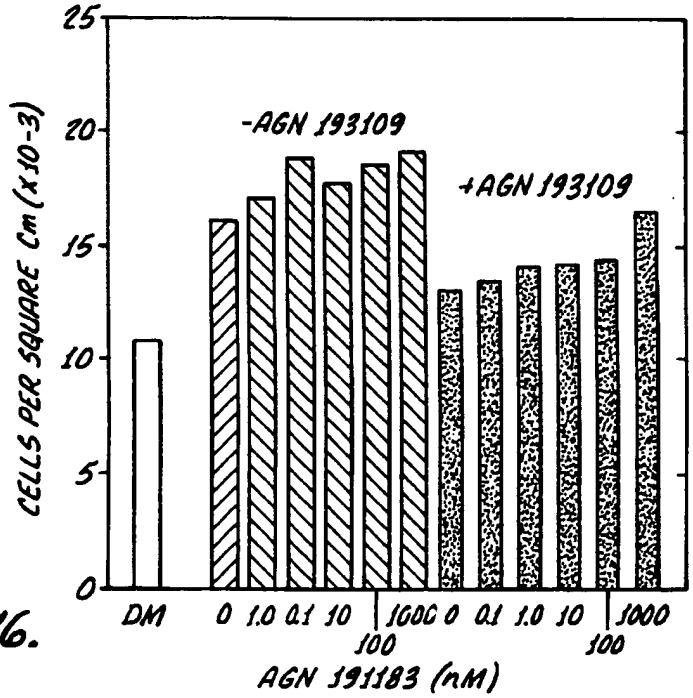

25
20
15
10
5
0
CELLS PER SQUARE Cm (x10-3)
-AGN 193109
+AGN 193109
DM 0 1.0 0.1 10 100 0 0.1 1.0 10 100
AGN 191183 (nM)

FIG. 16.

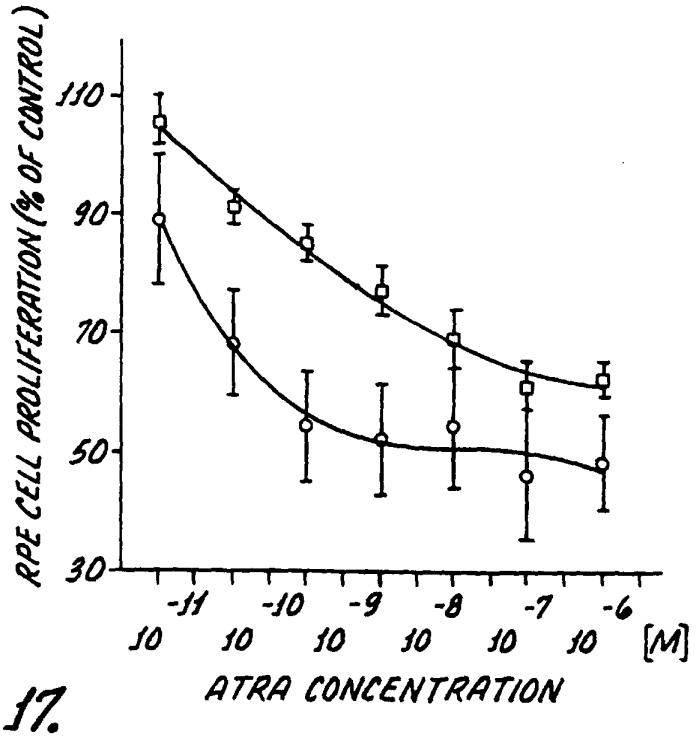

RPE CELL PROLIFERATION (% OF CONTROL)
110
90
70
50
30
-11 -10 -9 -8 -7 -6
10 10 10 10 10 10 [M]
ATRA CONCENTRATION

FIG. 17.

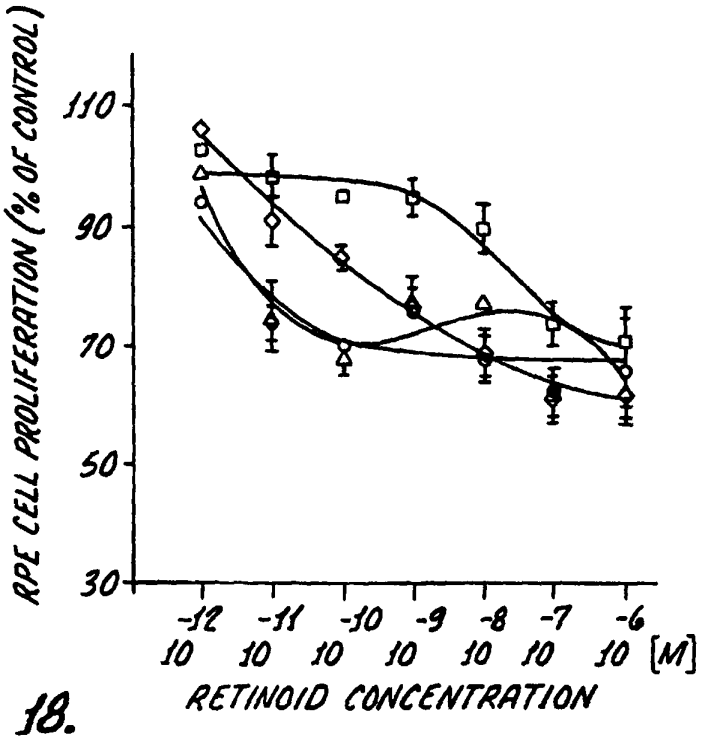

RPE CELL PROLIFERATION (% OF CONTROL)
110
90
70
50
30
-12 -11 -10 -9 -8 -7 -6
10 10 10 10 10 10 10 [M]
RETINOID CONCENTRATION

FIG. 18.

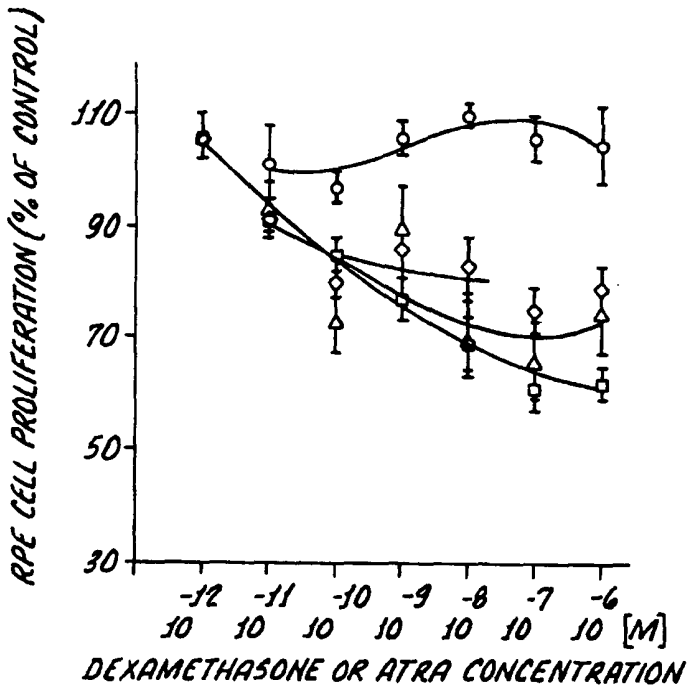

RPE CELL PROLIFERATION (% OF CONTROL)
110
90
70
50
30
-12 -11 -10 -9 -8 -7 -6
10 10 10 10 10 10 10 [M]
DEXAMETHASONE OR ATRA CONCENTRATION

FIG. 19.

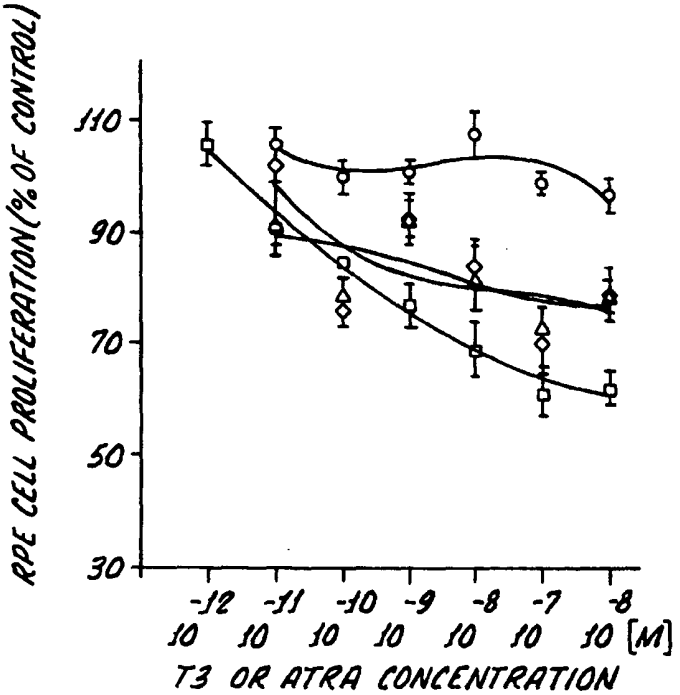

RPE CELL PROLIFERATION (% OF CONTROL)
110
90
70
50
30
-12 -11 -10 -9 -8 -7 -8
10 10 10 10 10 10 10 [M]
T3 OR ATRA CONCENTRATION

FIG. 20.

SYNTHESIS AND USE OF RETINOID COMPOUNDS HAVING NEGATIVE HORMONE AND/OR ANTAGONIST ACTIVITIES

RELATED APPLICATIONS

This application is a divisional of application Ser. No. 08\871,093 filed on Jun. 9, 1997 now U.S. Pat. No. 5,952,345, which is a divisional of application Ser. No. 08/613,863 filed on Mar. 11, 1996, now U.S. Pat. No. 5,776,699, which claims the benefit of priority under 35 U.S.C. § 119(e) of the three following U.S. applications, each of which was filed as a nonprovisional application and converted to a provisional application by separate petitions filed on Jan. 31, 1996: application Ser. No. 08/522,778, filed Sep. 1, 1995, now provisional application Ser. No. 60/019,015 application Ser. No. 08/522,778, filed Sep. 1, 1995, now provisional application Ser. No. 60/064,853; and application Ser. No. 08/542,648, filed Oct. 13, 1995, now provisional application Ser. No. 60/020,501. The complete disclosures of these related applications is hereby incorporated herein by this reference thereto.

FIELD OF THE INVENTION

The present invention relates to novel compounds having retinoid negative hormone and/or retinoid antagonist-like biological activities. More specifically, the invention relates to 4-aryl substituted benzopyran, 4-aryl substituted benzothiopyran, 4-aryl substituted 1,2-dihydroquinoline and 8-aryl substituted 5,6-dihydronaphthalene derivatives which may also be substituted by a substituted 3-oxo-1-propenyl group. These novel compounds have retinoid antagonist like-activity and are useful for treating or preventing retinoid and vitamin A and vitamin A precursor induced toxicity in mammals and as an adjunct to treatment of mammals with retinoids to prevent or ameliorate unwanted or undesired side effects. The invention further relates to the use of retinoid negative hormones for increasing the biological activities of other retinoids and steroid hormones and inhibiting the basal activity of unliganded retinoic acid receptors.

BACKGROUND OF THE INVENTION

Compounds which have retinoid-like activity are well known in the art, and are described in numerous United States and other patents and in scientific publications. It is generally known and accepted in the art that retinoid-like activity is useful for treating mammals, including humans, in order to cure or alleviate the symptoms associated with numerous diseases and conditions.

Retinoids (vitamin A and its derivatives) are known to have broad activities, including effects on cell proliferation and differentiation, in a variety of biological systems. This activity has made retinoids useful in the treatment of a variety of diseases, including dermatological disorders and cancers. The prior art has developed a large number of chemical compounds which have retinoid-like biological activity, and voluminous patent and chemical literature exists describing such compounds. The relevant patent literature includes U.S. Pat. Nos. 4,980,369, 5,006,550, 5,015,658, 5,045,551, 5,089,509, 5,134,159, 5,162,546, 5,234,926, 5,248,777, 5,264,578, 5,272,156, 5,278,318, 5,324,744, 5,346,895, 5,346,915, 5,348,972, 5,348,975, 5,380,877, 5,399,561, 5,407,937, (assigned to the same assignee as the present application) and patents and publications cited therein, which particularly describe or relate to chroman, thiochroman and 1,2,3,4-tetrahydroquinoline derivatives

which have retinoid-like biological activity. In addition, several applications are pending which are assigned to the assignee of the present application, and which are directed to further compounds having retinoid-like activity.

U.S. Pat. Nos. 4,740,519 (Shroot et al.), 4,826,969 (Maignan et al.), 4,326,055 (Loeliger et al.), 5,130,335 (Chandraratna et al.), 5,037,825 (Klaus et al.), 5,231,113 (Chandraratna et al.), 5,324,840 (Chandraratna), 5,344,959 (Chandraratna), 5,130,335 (Chandraratna et al.), Published European Patent Application Nos. 0 176 034 A (Wuest et al.), 0 350 846 A (Klaus et al.), 0 176 032 A (Frickel et al.), 0 176 033 A (Frickel et al.), 0 253 302 A (Klaus et al.), 0 303 915 A (Bryce et al.), UK Patent Application GB 2190378 A (Klaus et al.), German Patent Application Nos. DE 3715955 Al (Klaus et al.), DE 3602473 Al (Wuest et al., and the articles *J Amer. Acad. Derm.* 15: 756–764 (1986) (Sporn et al.), *Chem. Pharm. Bull.* 33: 404–407 (1985) (Shudo et al.), *J. Med Chem.* 31: 2182–2192 (1988) (Kagechika et al.), *Chemistry and Biology of Synthetic Retinoids* CRC Press Inc. 1990 pp. 334–335, 354 (Dawson et al.), describe or relate to compounds which include a tetrahydronaphthyl moiety and have retinoid-like or related biological activity. U.S. Pat. No. 4,391,731 (Boller et al.) describes tetrahydronaphthalene derivatives which are useful in liquid crystal compositions.

An article by Kagechika et al. in *J. Med Chem* 32:834 (1989) describe certain 6-(3-oxo-1-propenyl)-1,2,3,4-tetramethyl-1,2,3,4-tetrahydronaphthalene derivatives and related flavone compounds having retinoid-like activity. The articles by Shudo et al. in *Chem. Pharm. Bull.* 33:404 (1985) and by Jetten et al. in *Cancer Research* 47:3523 (1987) describe or relate to further 3-oxo-1-propenyl derivatives (chalcone compounds) and their retinoid-like or related biological activity.

Unfortunately, compounds having retinoid-like activity (retinoids) also cause a number of undesired side effects at therapeutic dose levels, including headache, teratogenesis, mucocutaneous toxicity, musculoskeletal toxicity, dyslipidemias, skin irritation, headache and hepatotoxicity. These side effects limit the acceptability and utility of retinoids for treating disease.

It is now general knowledge in the art that two main types of retinoid receptors exist in mammals (and other organisms). The two main types or families of receptors are respectively designated as the RARs and RXRs. Within each type there are subtypes: in the RAR family the subtypes are designated RAR-α, RAR-β and RAR-γ, in RXR the subtypes are: RXR-α, RXB-β and RXR-γ. Both families of receptors are transcription factors that can be distinguished from each other based on their ligand binding specificities. All-trans-RA (ATRA) binds and activates a class of retinoic acid receptors (RARs) that includes RAR-α, RAR-β and RAR-γ. A different ligand, 9-cis-RA (9C-RA), binds and activates both the RARs and members of the retinoid X receptor (RXR) family.

It has also been established in the art that the distribution of the two main retinoid receptor types, and of the several subtypes is not uniform in the various tissues and organs of mammalian organisms. Moreover, it is generally accepted in the art that many unwanted side effects of retinoids are mediated by one or more of the RAR receptor subtypes. Accordingly, among compounds having agonist-like activity at retinoid receptors, specificity or selectivity for one of the main types or families, and even specificity or selectivity for one or more subtypes within a family of receptors, is considered a desirable pharmacological property.

Relatively recently compounds have been developed in the art which bind to RAR receptors without triggering the response or responses that are triggered by agonists of the same receptors. The compounds or agents which bind to RAR receptors without triggering a "retinoid" response are thus capable of blocking (to lesser or greater extent) the activity of RAR agonists in biological assays and systems. More particularly, regarding the scientific and patent literature in this field, published PCT Application WO 94/14777 describes certain heterocyclic carboxylic acid derivatives which bind to RAR retinoid receptors and are said in the application to be useful for treatment of certain diseases or conditions, such as acne, psoriasis, rheumatoid arthritis and viral infections. A similar disclosure is made in the article by Yoshimura et al. *J. Med. Chem.* 38: 3163–3173 (1995). Kaneko et al. *Med. Chem Res.* 1:220–225 (1991); Apfel et al. *Proc. Natl. Acad. Sci. USA* 89: 7129–7133 Augusty 1992 *Cell Biology;* Eckhardt et al. *Toxicology Letters* 70:299–308 (1994); Keidel et al. *Molecular and Cellular Biology* 14:287–298 (1994); and Eyrolles et al. *J. Med. Chem.* 37: 1508–1517 (1994) describe compounds which have antagonist like activity at one or more of the RAR retinoid subtypes.

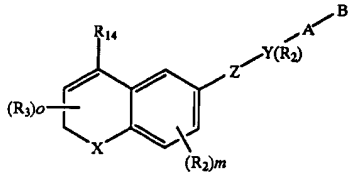
In addition to undesirable side-effects of therapy with retinoid compounds, there occurs occasionally a serious medical condition caused by vitamin A or vitamin A precursor overdose, resulting either from the excessive intake of vitamin supplements or the ingestion of liver of certain fish and animals that contain high levels of the vitamin. The chronic or acute toxicities observed with hypervitaminosis A syndrome include headache, skin peeling, bone toxicity, dyslipidemias, etc. In recent years, it has become apparent that the toxicities observed with vitamin A analogs, i.e., retinoids, essentially recapitulate those of hypervitaminosis A syndrome, suggesting a common biological cause, i.e., RAR activation. These toxicities are presently treated mainly by supportive measures and by abstaining from further exposure to the causative agent, whether it be liver, vitamin supplements, or retinoids. While some of the toxicities resolve with time, others (e.g., premature epiphyseal plate closure) are permanent.

Generally speaking, specific antidotes are the best treatment for poisoning by pharmacological agents, but only about two dozen chemicals or classes of chemicals out of thousands in existence have specific known antidotes. A specific antidote would clearly be of value in the treatment of hypervitaminosis A and retinoid toxicity. Indeed, as increasingly potent retinoids are used clinically, a specific antidote for retinoid poisoning could be life saving.

SUMMARY OF THE INVENTION

The present invention covers compounds of Formula 1

Formula 1



wherein

X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons, or

X is $[C(R_1)_2]_n$ where R_1 is independently H or alkyl of 1 to 6 carbons, and n is an integer between 0 and 2;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C≡C—,

—N=N—,

—N=CR_1—,

—CR_1=N,

—$(CR_1=CR_1)_{n'}$— where n' is an integer having the value 0–5,

—CO—NR_1—,

—CS—NR_1—,

—NR_1—CO,

—NR_1—CS,

—COO—,

—OCO—;

—CSO—;

—OCS—;

Y is a phenyl or naphthyl group, or heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said phenyl and heteroaryl groups being optionally substituted with one or two R_2 groups, or when Z is $—(CR_1=CR_1)_{n'}—$ and n' is 3, 4 or 5 then Y represents a direct valence bond between said $(CR_2=CR_2)_{n'}$ group and B;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

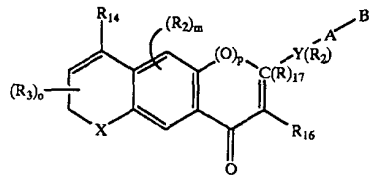
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, —CH_2OH, CH_2R_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, —COR_7, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$-phenyl, $(R_{15})_r$-naphthyl, or $(R_{15})_r$-heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $N(R_8)COR_8$, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

The present invention further covers compounds of Formula 101

Formula 101



wherein

X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons, or

X is $[C(R_1)_2]_n$ where R_1 is independently H or alkyl of 1 to 6 carbons, and n is an integer between 0 and 2;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is a phenyl or naphthyl group, or heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said phenyl and heteroaryl groups being optionally substituted with one or two R_2 groups;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$-phenyl, $(R_{15})_r$-naphthyl, or $(R_{15})_r$-heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $N(R_8)COR_8$, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons;

R_{16} is H, lower alkyl of 1 to 6 carbons;

R_{17} is H, lower alkyl of 1 to 6 carbons, OH or $OCOR_{11}$, and

p is zero or 1, with the proviso that when p is 1 then there is no R_{17} substituent group, and m is an integer between 0 and 2.

The compounds of the present invention are useful for preventing certain undesired side effects of retinoids which are administered for the treatment or prevention of certain diseases or conditions. For this purpose the compounds of the invention may be coadministered with retinoids. The compounds of the present invention are also useful in the treatment of acute or chronic toxicity resulting from overdose or poisoning by retinoid drugs or Vitamin A.

The present invention additionally relates to the use of RAR antagonists for blocking all or some RAR receptor sites in biological systems, including mammals, to prevent or diminish action of RAR agonists on said receptor sites. More particularly, the present invention relates to the use of RAR antagonists for (a) the prevention and (b) the treatment of retinoid (including vitamin A or vitamin A precursor) chronic or acute toxicity and side effects of retinoid therapy.

In one particular aspect of the present invention, there is provided a method of treating a pathological condition in a mammal. The conditions treated are associated with a retinoic acid receptor activity. This method involves administering to the mammal a retinoid antagonist or negative hormone capable of binding to one of the following retinoic acid receptor subtypes: RAR_α, RAR_β and RAR_γ. The antagonist or negative hormone is administered in an amount pharmaceutically effective to provide a therapeutic benefit against the pathological condition in the mammal.

As an antidote to acute or chronic retinoid or vitamin A poisoning the RAR antagonist can be administered to a mammal enterally, i.e., intragastric intubation or food/water admixture, or parenterally, e.g., intraperitoneally, intramuscularly, subcutaneously, topically, etc. The only requirement for the route of administration is that it must allow delivery of the antagonist to the target tissue. The RAR antagonist can be formulated by itself or in combination with excipients. The RAR antagonist need not be in solution in the formulation, e.g., in the case of enteral use.

As an adjunct to therapy with retinoids and in order to prevent one or more side effects of the retinoid drug which is administered, the RAR antagonist can similarly be administered enterally or parenterally. The RAR antagonist and RAR agonist need not be administered by the same route of administration. The key is that sufficient quantities of the RAR antagonist be present continuously in the tissue of interest during exposure to the RAR agonist. For the prevention of retinoid toxicity, it is best that the RAR antagonist be administered concurrently or prior to treatment with the RAR agonist. In many situations the RAR antagonist will be administered by a different route than the agonist. For example undesirable skin effects of an enterally administered retinoid may be prevented or ameliorated by an RAR antagonist which is administered topically.

Another aspect of the present invention is a method of identifying retinoid negative hormones. The method includes the following steps: obtaining transfected cells containing a reporter gene transcriptionally responsive to binding of a recombinant retinoid receptor, the recombinant retinoid receptor having at least protein domains located C-terminal to a DNA binding domain of an intact retinoid receptor, measuring a basal level of reporter gene expression in untreated transfected cells, the untreated transfected cells being propagated in the absence of an added retinoid, treating the transfected cells with a retinoid compound to be tested for negative hormone activity, measuring a level of reporter gene expression in treated cells, comparing the levels of reporter gene expression measured in treated cells and untreated cells, and identifying as retinoid negative hormones those retinoid compounds producing a lower level of reporter gene expression in treated cells compared with the basal level of reporter gene expression measured in untreated cells. In certain preferred embodiments of this method the intact receptor is an RAR-α, RAR-β or RAR-γ subtype. In other embodiments, the intact receptor is an

RXR-α, RXR-β or RXR-γ subtype. The recombinant receptor can also be either a recombinant RAR or RXR receptor. In some embodiments, the recombinant receptor is a chimeric retinoid receptor having a constitutive transcription activator domain. Such a constitutive transcription activator domain can comprise a plurality of amino acids having a net negative charge or have an amino acid sequence of a viral transcription activator domain, such as the herpes simplex virus VP-16 transcription activator domain. In embodiments in which the constitutive transcription activator domain has a net negative charge, the retinoid receptor can be recombinant and have deleted therefrom a DNA binding domain, such as a DNA binding domain specific for a cis-regulatory element other than a retinoic acid responsive element. These elements include an estrogen responsive element. The transfected cell is preferably propagated in a growth medium substantially depleted of endogenous retinoids, such as one that includes activated charcoal-extracted serum. In this method, the reporter gene can be the luciferase gene, in which case, the measuring steps can involve luminometry. The reporter gene can also be the β-galactosidase gene, in which case the measuring steps would involve a β-galactosidase assay. The transfected cell can be a transfected mammalian cell, such as a Green monkey cell or a human cell.

Another aspect of the present invention is a method of potentiating a pharmacologic activity of a steroid superfamily receptor agonist administered to a mammal. This method involves coadministering to the mammal with the steroid superfamily receptor agonist a composition comprising a pharmaceutically effective dose of a retinoid negative hormone to potentiate the pharmacologic activity of the steroid superfamily receptor agonist. The pharmacologic activity is measurable in a reporter gene trans-activation assay in vitro, such as by measuring anti-AP-1 activity. The pharmacologic activity to be potentiated can be an antiproliferative activity, such as activity of the type measurable in retinal pigment epithelium. The steroid superfamily receptor agonist can be any of the following: a retinoid receptor agonist, a vitamin D receptor agonist, a glucocorticoid receptor agonist, a thyroid hormone receptor agonist, a peroxisome proliferator-activated receptor agonist or an estrogen receptor agonist. The retinoid receptor agonist can be an RAR agonist, such as all-trans-retinoic acid or 13-cis retinoic acid. The retinoid receptor agonist can also be an RXR agonist. A preferred vitamin D receptor agonist is 1,25-dihydroxyvitamin D_3. A preferred glucocorticoid receptor agonist is dexamethasone. A preferred thyroid hormone receptor agonist is 3,3',5-triiodothyronine. The retinoid negative hormone is an RAR-specific retinoid negative hormone, which preferably has a dissociation constant less than or approximately equal to 30 nM. Example of the RAR-specific retinoid negative hormone include AGN 193109, AGN 193385, AGN 193389 and AGN 193871. The composition comprising a pharmaceutically effective dose of a retinoid negative hormone can be coadministered at the same time as the steroid superfamily agonist and be combined prior to coadministration. These can also be coadministered as separate compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the chemical structure of AGN 193109.

FIGS. **2A–2F** are a series of line graphs showing that AGN 193109 inhibited ATRA-dependent transactivation at the RARs. FIGS. **2A** and **2B** represent activity at the RAR-α receptor; FIGS. **2C** and **2D** represent activity at the RAR-β receptor; FIGS. **2E** and **2F** represent activity at the RAR-γ receptor. In FIGS. **2A**, **2C** and **2E**, open squares represent retinoic acid treatment and filled circles represent AGN 193109 treatment. In FIGS. **2B**, **2D** and **2F** the single lines represent luciferase activity measured after treatment with 10^{-8} M ATRA and variable concentrations of AGN 193109.

FIGS. **3A** and **3B** are line graphs representing luciferase activity detected in CV-1 cells transfected with reporter plasmid ERE-tk-Luc and expression plasmid ER-RAR-α and stimulated with ATRA (FIG. **3A**) or AGN 193109 (FIG. **3B**) at various concentrations. Data points represent the mean ±SEM of three independent luciferase determinations. The results of transfections carried out using different amounts of co-transfected ER-RAR-α (0.05, 0.1 and 0.2 μg/well) are indicated in each figure.

FIGS. **4A** and **4B** are line graphs representing luciferase activity in CV-1 cells transfected with reporter plasmid ERE-tk-Luc and expression plasmid ER-RAR-β and stimulated with ATRA (FIG. **4A**) or AGN 193109 (FIG. **4B**) at various concentrations. Data points represent the mean ±SEM of three independent luciferase determinations. The results of transfections carried out using different amounts of co-transfected ER-RAR-β (0.05, 0.1 and 0.2 μg/well) are indicated in each figure.

FIGS. **5A** and **5B** are line graphs representing luciferase activity detected in CV-1 cells transfected with reporter plasmid ERE-tk-Luc and expression plasmid ER-RAR-γ and stimulated with ATRA (FIG. **5A**) or AGN 193109 (FIG. **5B**) at various concentrations. Data points represent the mean ±SEM of three independent luciferase determinations. The results of transfections carried out using different amounts of co-transfected ER-RAR-γ (0.05, 0.1 and 0.2 μg/well) are indicated in each figure.

FIG. **6** shows ATRA and AGN 193109 dose responses of CV-1 cells cotransfected with the ERE-tk-Luc reporter plasmid and either the ER-RXR-α chimeric receptor expression plasmid alone, or in combination with the RAR-γ-VP-16 expression plasmid. ER-RXR-α cotransfected cells were treated with ATRA (square) and AGN 193109 (diamond). Cells cotransfected with the combination of ER-RXR-α and RAR-γ-VP-16 were treated with ATRA (circle) or AGN 193109 (triangle).

FIG. **7** shows a line graph representing luciferase activity measurements recorded in lysates of CV-1 cells transfected with the ERE-tk-Luc reporter and ER-RAR-γ expression construct and then treated with ATRA at 10^{-8} M and the test compounds at the concentrations indicated on the horizontal axis. The test compounds were AGN 193109 (square), AGN 193357 (open diamond), AGN 193385 (circle), AGN 193389 (triangle), AGN 193840 (hatched square) and AGN 192870 (filled diamond).

FIG. **8** shows a line graph representing luciferase activity measurements recorded in lysates of CV-1 cells transfected with the ERE-tk-Luc reporter and RAR-γ-VP-16 and ER-RXR-α expression constructs and then treated with the test compounds at the concentrations indicated on the horizontal axis. The test compounds were ATRA (open square), AGN 193109 (open circle), AGN 193174 (open triangle), AGN 193199 (hatched square), AGN 193385 (hatched circle), AGN 193389 (inverted triangle), AGN 193840 (diagonally filled square) and AGN 193871 (half-filled diamond).

FIGS. **9A**, **9B** and **9C** schematically diagram a mechanism whereby AGN 193109 can modulate the interaction between the RAR (shaded box) and negative coactivator proteins (–) illustrated in the context of a transactivation assay. FIG. **9A** shows that negative coactivator proteins and

positive coactivator proteins (+) are in a binding equilibrium with the RAR. In the absence of a ligand, basal level transcription of the reporter gene results. As illustrated in FIG. 9B, addition of an RAR agonist promotes the association of positive coactivator proteins with the RAR and results in upregulated reporter gene transcription. As illustrated in FIG. 9C, addition of AGN 193109 promotes the association of negative coactivator proteins with the RAR and prevents reporter gene transcription.

FIG. **10** is a bar graph showing the inhibition of TPA-induced Str-AP1-CAT expression as a function of AGN 191183 concentration (10^{-10} to 10^{-12} M) with the AGN 193109 concentration held constant at 10^{-8} M. Results from trials conducted with AGN 191183 alone are shown as hatched bars while stripped bars represent the results from treatment with the combination of AGN 193109 and AGN 191183.

FIG. **11** schematically diagrams a mechanism whereby AGN 193109 can potentiate the activities of RARs and other nuclear receptor family members. As illustrated in the diagram, introduced RARs (open rectangles having AB-C-DEF domains) have increased sensitivity to RAR ligands in the anti-AP1 assay because the negative coactivator protein (ncp), present in limiting supply, is sequestered onto RARs thereby leading to two populations: RAR+ncp and RAR−ncp. RAR−ncp has increased sensitivity to ligands. Non-RAR nuclear factors (shaded rectangles having AB-C-DEF domains) have increased sensitivity to cognate ligands because ncp has been sequestered to the RAR by the activity of AGN 193109. The modular domains of the nuclear receptors are designated using standard nomenclature as "AB" (ligand independent transactivation domain), "C" (DNA binding domain), and "DEF" (ligand regulated transactivation domain and dimerization domain.

FIG. **12** is a line graph showing the effect of AGN 193109 on the 1,25-dihydroxyvitamin D_3 dose response in CV-1 cells transfected with the MTV-DR3-Luc reporter plasmid. Transfectants were treated with 1,25-dihydroxyvitamin D_3 (filled square), 1,25-dihydroxyvitamin D_3 and 10^{-8} M AGN 193109 (filled triangle), and 1,25-dihydroxyvitamin D_3 and 10^{-7} M AGN 193109 (filled circle).

FIG. **13** is a bar graph showing the effect of AGN 193109 (10 nM) coadministration on 1,25-dihydroxyvitamin D_3-mediated inhibition of TPA induced Str-AP1-CAT activity. Filled bars represent inhibition of CAT activity in transfected cells treated with 1,25-dihydroxyvitamin D_3 alone. Open bars represent inhibition of CAT activity in transfected cells treated with the combination of 1,25-dihydroxyvitamin D_3 and AGN 193109.

FIG. **14** is a line graph showing the effect of AGN 193109 alone and in combination with AGN 191183 on HeLa cells cotransfected with RAR-γ and the RAR responsive MTV-TREp-Luc reporter construct. Drug treatments illustrated in the graph are: AGN 193109 alone (square), AGN 193109 in combination with AGN 191183 at 10^{-10} M (diamond) and AGN 193109 in combination with AGN 191183 at 10^{-9} M.

FIG. **15** is a line graph showing that ECE16-1 cells proliferated in response to EGF (filled square) but not in response to defined medium alone (open circle). Cells treated with AGN 193109 alone are represented by the filled triangle. The filled circles represent results obtained for cells treated with 10 nM AGN 191183 and 0–1000 nM AGN 193109.

FIG. **16** is a bar graph showing the effect of AGN 193109 on the proliferation of CaSki cells in the presence or absence of the AGN 191183 retinoid agonist. All sample groups received 20 ng/ml of epidermal growth factor (EGF) with the exception of the sample propagated in defined medium (DM) alone (open bar). Stripped bars represent samples propagated in the absence of AGN 193109. Filled bars represent samples propagated in the presence of 1000 nM AGN 193109. The concentrations of AGN 191183 used in the procedure are shown on the horizontal axis.

FIG. **17** is a dose response curve showing that AGN 193109 potentiated the antiproliferative activity of ATRA on retinal pigment epithelium (RPE) cells. Samples treated with ATRA alone are represented by filled squares. Samples treated with the combination of ATRA and AGN 193109 (10^{-7} M) are represented by filled circles. The ATRA concentration used for treating the various samples is given on the horizontal axis.

FIG. **18** is a dose response curve showing that both 13-cis-RA and ATRA inhibited RPE cell growth, and that AGN 193109 potentiated the antiproliferative activity of 13-cis-RA. The various sample treatments shown in the dose response included 13-cis-RA alone (filled square), 13-cis-RA in combination with AGN 193109 (10^{-6} M) (filled circle), 13-cis-RA in combination with AGN 193109 (10^{-8} M) (filled triangle), and ATRA (filled diamond). The concentrations of 13-cis-RA and ATRA used in the sample treatments are shown on the horizontal axis.

FIG. **19** is a dose response curve showing that AGN 193109 potentiated the antiproliferative activity of dexamethasone in primary RPE cell cultures. The various sample treatments shown in the dose response included ATRA (filled square), dexamethasone alone (filled circle), dexamethasone in combination with AGN 193109 (10^{-8} M) (filled triangle), and dexamethasone in combination with AGN 193109 (10^{-6} M) (filled diamond). The concentrations of dexamethasone and ATRA used in the sample treatments are shown on the horizontal axis.

FIG. **20** is a dose response curve showing that AGN 193109 potentiated the antiproliferative activity of thyroid hormone (T3) in primary RPE cell cultures. The various sample treatments shown in the dose response included ATRA (filled square), T3 alone (filled circle), T3 in combination with AGN 193109 (10^{-8} M) (filled triangle), T3 in combination with AGN 193109 (10^{-6} M) (filled diamond). The concentrations of T3 and ATRA used in the sample treatments are shown on the horizontal axis.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

For the purposes of the present invention, an RAR antagonist is defined as a chemical that binds to one or more of the RAR subtypes with a K_d of less than 1 micromolar (K_d<1 μM) but which does not cause significant transcriptional activation of that RAR subtypes-regulated genes in a receptor co-transfection assay. Conventionally, antagonists are chemical agents that inhibit the activities of agonists. Thus, the activity of a receptor antagonist is conventionally measured by virtue of its ability to inhibit the activity of an agonist.

An RAR agonist is defined as a chemical that binds to one or more RAR receptor subtype with K_d of less than 1 micromol (K_d<1 μM) and causes transcriptional activation of that RAR-subtype-regulated genes in a receptor co-transfection assay. The term "RAR agonist" includes chemicals that may bind and/or activate other receptors in addition to RARs, e.g., RXR receptors.

As used herein, a negative hormone or inverse agonist is a ligand for a receptor which causes the receptor to adopt an inactive state relative to a basal state occurring in the absence of any ligand. Thus, while an antagonist can inhibit the activity of an agonist, a negative hormone is a ligand that can alter the conformation of the receptor in the absence of an agonist. The concept of a negative hormone or inverse agonist has been explored by Bond et al. in *Nature* 374:272 (1995). More specifically, Bond et al. have proposed that unliganded β_2-adrenoceptor exists in an equilibrium between an inactive conformation and a spontaneously active conformation. Agonists are proposed to stabilize the receptor in an active conformation. Conversely, inverse agonists are believed to stabilize an inactive receptor conformation. Thus, while an antagonist manifests its activity by virtue of inhibiting an agonist, a negative hormone can additionally manifest its activity in the absence of an agonist by inhibiting the spontaneous conversion of an unliganded receptor to an active conformation. Only a subset of antagonists will act as negative hormones. As disclosed herein, AGN 193109 is both an antagonist and a negative hormone. To date, no other retinoids have been shown to have negative hormone activity.

As used herein, coadministration of two pharmacologically active compounds refers to the delivery of two separate chemical entities, whether in vitro or in vivo. Coadministration refers to the simultaneous delivery of separate agents; to the simultaneous delivery of a mixture of agents; as well as to the delivery of one agent followed by delivery of the second agent. In all cases, agents that are coadministered are intended to work in conjunction with each other.

The term alkyl refers to and covers any and all groups which are known as normal alkyl, branched-chain alkyl and cycloalkyl. The term alkenyl refers to and covers normal alkenyl, branch chain alkenyl and cycloalkenyl groups having one or more sites of unsaturation. Similarly, the term alkynyl refers to and covers normal alkynyl, and branch chain alkynyl groups having one or more triple bonds.

Lower alkyl means the above-defined broad definition of alkyl groups having 1 to 6 carbons in case of normal lower alkyl, and as applicable 3 to 6 carbons for lower branch chained and cycloalkyl groups. Lower alkenyl is defined similarly having 2 to 6 carbons for normal lower alkenyl groups, and 3 to 6 carbons for branch chained and cyclo-lower alkenyl groups. Lower alkynyl is also defined similarly, having 2 to 6 carbons for normal lower alkynyl groups, and 4 to 6 carbons for branch chained lower alkynyl groups.

The term "ester" as used here refers to and covers any compound falling within the definition of that term as classically used in organic chemistry. It includes organic and inorganic esters. Where B (of Formula 1 or Formula 101) is —COOH, this term covers the products derived from treatment of this function with alcohols or thiols preferably with aliphatic alcohols having 1–6 carbons. Where the ester is derived from compounds where B is $—CH_2OH$, this term covers compounds derived from organic acids capable of forming esters including phosphorous based and sulfur based acids, or compounds of the formula $—CH_2OCOR_{11}$ where R_{11} is any substituted or unsubstituted aliphatic, aromatic, heteroaromatic or aliphatic aromatic group, preferably with 1–6 carbons in the aliphatic portions.

Unless stated otherwise in this application, preferred esters are derived from the saturated aliphatic alcohols or acids of ten or fewer carbon atoms or the cyclic or saturated aliphatic cyclic alcohols and acids of 5 to 10 carbon atoms. Particularly preferred aliphatic esters are those derived from lower alkyl acids and alcohols. Also preferred are the phenyl or lower alkyl phenyl esters.

Amides has the meaning classically accorded that term in organic chemistry. In this instance it includes the unsubstituted amides and all aliphatic and aromatic mono- and di-substituted amides. Unless stated otherwise in this application, preferred amides are the mono- and di-substituted amides derived from the saturated aliphatic radicals of ten or fewer carbon atoms or the cyclic or saturated aliphatic-cyclic radicals of 5 to 10 carbon atoms. Particularly preferred amides are those derived from substituted and unsubstituted lower alkyl amines. Also preferred are mono- and disubstituted amides derived from the substituted and unsubstituted phenyl or lower alkylphenyl amines. Unsubstituted amides are also preferred.

Acetals and ketals include the radicals of the formula-CK where K is $(—OR)_2$. Here, R is lower alkyl. Also, K may be $—OR_7O—$ where R_7 is lower alkyl of 2–5 carbon atoms, straight chain or branched.

A pharmaceutically acceptable salt may be prepared for any compounds in this invention having a functionality capable of forming a salt, for example an acid functionality. A pharmaceutically acceptable salt is any salt which retains the activity of the parent compound and does not impart any deleterious or untoward effect on the subject to which it is administered and in the context in which it is administered.

Pharmaceutically acceptable salts may be derived from organic or inorganic bases. The salt may be a mono or polyvalent ion. Of particular interest are the inorganic ions, sodium, potassium, calcium, and magnesium. Organic salts may be made with amines, particularly ammonium salts such as mono-, di- and trialkyl amines or ethanol amines. Salts may also be formed with caffeine, tromethamine and similar molecules. Where there is a nitrogen sufficiently basic as to be capable of forming acid addition salts, such may be formed with any inorganic or organic acids or alkylating agent such as methyl iodide. Preferred salts are those formed with inorganic acids such as hydrochloric acid, sulfuric acid or phosphoric acid. Any of a number of simple organic acids such as mono-, di- or tri- acid may also be used.

Some of the compounds of the present invention may have trans and cis (E and Z) isomers. In addition, the compounds of the present invention may contain one or more chiral centers and therefore may exist in enantiomeric and diastereomeric forms. The scope of the present invention is intended to cover all such isomers per se, as well as mixtures of cis and trans isomers, mixtures of diastereomers and racemic mixtures of enantiomers (optical isomers) as well. In the present application when no specific mention is made of the configuration (cis, trans or R or S) of a compound (or of an asymmetric carbon) then a mixture of such isomers, or either one of the isomers is intended.

Aryl Substituted Benzopyran, Benzothiopyran, 1,2-Dihydroquinoline and 5,6-Dihydronaphthalene Derivatives Having Retinoid Antagonist Like Biological Activity

With reference to the symbol Y in Formula 1, the preferred compounds of the invention are those where Y is phenyl, pyridyl, thienyl or furyl. Even more preferred are compounds where Y is phenyl or pyridyl, and still more preferred where Y is phenyl. As far as substitutions on the Y (phenyl) and Y (pyridyl) groups are concerned, compounds are preferred where the phenyl group is 1,4 (para) substi-

tuted by the Z and A-B groups, and where the pyridine ring is 2,5 substituted by the Z and A-B groups. (Substitution in the 2,5 positions in the "pyridine" nomenclature corresponds to substitution in the 6-position in the "nicotinic acid" nomenclature.) In the preferred compounds of the invention either there is no optional R_2 substituent on the Y group, or the optional R_2 substituent is fluoro (F).

The A-B group of the preferred compounds is $(CH_2)_n$—COOH or $(CH_2)_n$—$COOR_8$, where n and R_8 are defined as above. Even more preferably n is zero and R_8 is lower alkyl, or n is zero and B is COOH or a pharmaceutically acceptable salt thereof.

In the majority of the presently preferred examples of compounds of the invention X is $[C(R_1)_2]_n$ where n is 1. Nevertheless, compounds where n is zero (indene derivatives) and where X is S or O (benzothiopyran and benzopyran derivatives) are also preferred. When X is $[C(R_1)_2]_n$ and n is 1, then R_1 preferably is alkyl of 1 to 6 carbons, even more preferably methyl.

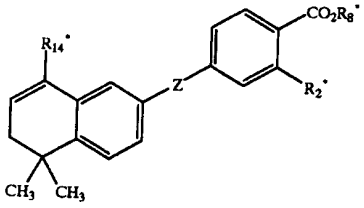
The R_2 group attached to the aromatic portion of the tetrahydronaphthalene, benzopyran, benzothiopyran or dihydroquinoline moiety of the compounds of Formula 1 is preferably H, F or CF_3. R_3 is preferably hydrogen or methyl, even more preferably hydrogen.

Referring now to the group Z in the compounds of the invention and shown in Formula 1, in a plurality of preferred examples Z represents an acetylenic linkage (Z═—C≡C—). However, the "linker group" Z is also preferred as a diazo group (Z═—N═N—), as an olefinic or polyolefinic group (Z═—$(CR_1{=}CR_1)_n$—), as an ester (Z═—COO—), amide (Z═—CO—NR_2—) or thioamide (Z═—CS—NR_2—) linkage.

Referring now to the R_{14} group, compounds are preferred where R_{14} is phenyl, 2-pyridyl, 3-pyridyl, 2-thienyl, and 2-thiazolyl. The R_{15} group (substituent of the R_{14} group) is preferably H, lower alkyl, trifluoromethyl, chlorine, lower alkoxy or hydroxy.

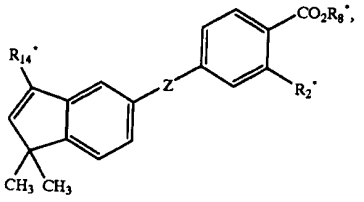
The presently most preferred compounds of the invention are shown in Table 1 with reference to Formula 2, Formula 3, Formula 4, Formula 5, and Formula 5a.

Formula 2

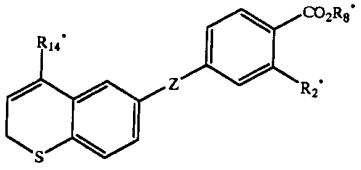


-continued

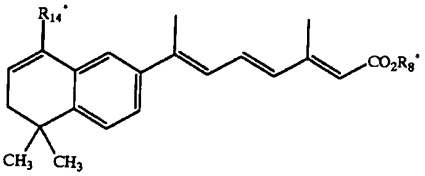
Formula 3



Formula 4



Formula 5



Formula 5a

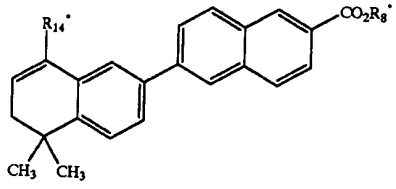


TABLE 1

Compound #	Formula	R_{14}*	Z	R_2*	R_8*
1	2	4-methylphenyl	—C≡C—	H	Et
1a	2	phenyl	—C≡C—	H	Et
2	2	3-methylphenyl	—C≡C—	H	Et
3	2	2-methylphenyl	—C≡C—	H	Et
4	2	3,5-dimethylphenyl	—C≡C—	H	Et
5	2	4-ethylphenyl	—C≡C—	H	Et
6	2	4-t-butylphenyl	—C≡C—	H	Et
7	2	4-chlorophenyl	—C≡C—	H	Et

TABLE 1-continued

Compound #	Formula	R_{14}*	Z	R_2*	R_8*
8	2	4-methoxyphenyl	—C≡C—	H	Et
9	2	4-trifluoromethylphenyl	—C≡C—	H	Et
10	2	2-pyridyl	—C≡C—	H	Et
11	2	3-pyridyl	—C≡C—	H	Et
12	2	2-methyl-5-pyridyl	—C≡C—	H	Et
13	2	3-hydroxyphenyl	—C≡C—	H	Et
14	2	4-hydroxy phenyl	—C≡C—	H	Et
15	2	5-methyl-2-thiazolyl	—C≡C—	H	Et
15a	2	2-thiazolyl	—C≡C—	H	Et
16	2	4-methyl-2-thiazolyl	—C≡C—	H	Et
17	2	4,5-dimethyl-2-thiazolyl	—C≡C—	H	Et
18	2	2-methyl-5-pyridyl	—C≡C—	H	H
19	2	2-pyridyl	—C≡C—	H	H
20	2	3-methylphenyl	—C≡C—	H	H
21	2	4-ethylphenyl	—C≡C—	H	H
22	2	4-methoxyphenyl	—C≡C—	H	H
23	2	4-trifluoromethylphenyl	—C≡C—	H	H
24	2	3,5-dimethylphenyl	—C≡C—	H	H
25	2	47chlorophenyl	—C≡C—	H	H
26	2	3-pyndyl	—C≡C—	H	H
27	2	2-methylphenyl	—C≡C—	H	H
28	2	3-hydroxyphenyl	—C≡C—	H	H
29	2	4-hydroxyphenyl	—C≡C—	H	H
30	2	5-methyl-2-thiazolyl	—C≡C—	H	H
30a	2	2-thiazolyl-	—C≡C—	H	H
31	2	4-methyl-2-thiazolyl	—C≡C—	H	H
32	2	4,5-dimethyl-2-thiazolyl	—C≡C—	H	H
33	2	5-methyl-2-thienyl	—C≡C—	H	Et
33a	2	2-thienyl	—C≡C—	H	Et
34	2	5-methyl-2-thienyl	—C≡C—	H	H
34a	2	2-thienyl	—C≡C—	H	H
35	2	4-methylphenyl	—CONH—	H	Et
36	2	4-methylphenyl	—CONH—	H	H
37	2	4-methylphenyl	—COO—	H	Et
38	2	4-methylphenyl	—COO—	H	$(CH_2)_2Si(CH_3)$
39	2	4-methylphenyl	—COO—	H	H
40	2	4-methylphenyl	—CONH—	F	Et
41	2	4-methylphenyl	—CONH	F	H
42	2	4-methylphenyl	—CSNH—	H	Et
43	2	4-methylphenyl	—CSNH—	H	H
44	2	4-methylphenyl	—CH=CH—	H	Et
45	2	4-methylphenyl	—CH=CH—	H	H
46a	2	4-methylphenyl	—N=N—	H	Et
46b	2	4-methylphenyl	—N=N—	H	H
47	3	4-methylphenyl	—C≡C—	H	Et
48	3	4-methylphenyl	—C≡C—	H	H
49	4	4-methylphenyl	—C≡C—	H	Et
50	4	4-methylphenyl	—C≡C—	H	H
51	5	4-methylphenyl	—	—	Et
52	5	4-methylphenyl	—	—	H
60	2	4-methylphenyl	—C≡C—	H	H
60a	2	phenyl	—C≡C—	H	H
61	2	4-t-butylphenyl	—C≡C—	H	H
62	2	4-methylphenyl	—CSNH	F	Et
63	2	4-methylphenyl	—CSNH	F	H
64	5a	4-methylphenyl	—	—	Et
65	5a	4-methylphenyl	—	—	H
66	2	2-furyl	—C≡C—	H	Et
67	2	2-furyl	—C≡C—	H	H

Aryl and (3-Oxo-1-Propenyl)-Substituted Benzopyran, Benzothiopyran, Dihydroquinoline and 5,6-Dihydronaphthalene Derivatives Having Retinoid Antagonist-Like Biological Activity

With reference to the symbol Y in Formula 101, the preferred compounds of the invention are those where Y is phenyl, pyridyl, thienyl or furyl. Even more preferred are compounds where Y is phenyl or pyridyl, and still more preferred where Y is phenyl. As far as substitutions on the Y (phenyl) and Y (pyridyl) groups are concerned, compounds are preferred where the phenyl group is 1,4 (para) substituted by the $—CR_{16}=CR_{17}—$ and A-B groups, and where the pyridine ring is 2,5 substituted by the $—CR_{16}=CR_{17}—$ and A-B groups. (Substitution in the 2,5 positions in the "pyridine" nomenclature corresponds to substitution in the 6-position in the "nicotinic acid" nomenclature.) In the preferred compounds of the invention there is no optional R_2 substituent on the Y group.

The A-B group of the preferred compounds is $(CH_2)_n—COOH$ or $(CH_2)_n—COOR_8$, where n and R_8 are defined as above. Even more preferably n is zero and R_8 is lower alkyl, or n is zero and B is COOH or a pharmaceutically acceptable salt thereof.

In the presently preferred examples of compounds of the invention X is $[C(R_1)_2]_n$ where n is 1. Nevertheless, com-

pounds where X is S or O (benzothiopyran and benzopyran derivatives) are also preferred. When X is $[C(R_1)_2]_n$ and n is 1, then R_1 preferably is alkyl of 1 to 6 carbons, even more preferably methyl.

The R_2 group attached to the aromatic portion of the tetrahydronaphthalene, benzopyran, benzothiopyran or dihydroquinoline moiety of the compounds of Formula 101 is preferably H, F or CF_3. R_3 is preferably hydrogen or methyl, even more preferably hydrogen.

Referring now to the R_{14} group, compounds are preferred where R_{14} is phenyl, 2-pyridyl, 3-pyridyl, 2-thienyl, and 2-thiazolyl. The R_{15} group (substituent of the R_{14} group) is preferably H, lower alkyl, trifluoromethyl, chlorine, lower alkoxy or hydroxy.

Preferred compounds of the invention are shown in Table 2 with reference to Formula 102.

Formula 102

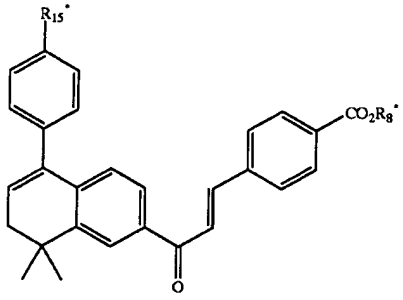


TABLE 2

Compound	R_{15}*	R_8*
101	CH_3	H
102	CH_3	Et
103	H	H
104	H	Et

Biological Activity, Modes of Administration

As noted above, the compounds of the present invention are antagonists of one or more RAR receptor subtypes. This means that the compounds of the invention bind to one or more RAR receptor subtypes, but do not trigger the response which is triggered by agonists of the same receptors. Some of the compounds of the present invention are antagonists of all three RAR receptor subtypes (RAR-α, RAR-β and RAR-γ), and these are termed "RAR pan antagonists". Some others are antagonists of only one or two of RAR receptor subtypes. Some compounds within the scope of the present invention are partial agonists of one or two RAR receptor subtypes and antagonists of the remaining subtypes. The compounds of the invention do not bind to RXR receptors, therefore they are neither agonists nor antagonists of RXR.

Depending on the site and nature of undesirable side effects which are desired to be suppressed or ameliorated, compounds used in accordance with the invention may be antagonists of only one or two of RAR receptor subtypes. Some compounds used in accordance with the invention may be partial agonists of one or two RAR receptor subtypes and antagonists of the remaining subtypes. Such compounds are, generally speaking, usable in accordance with the invention if the antagonist effect is on that RAR receptor subtype (or subtypes) which is (are) predominantly responsible for the overdose poisoning or for the undesired side effect or side effects. In this connection it is noted that, generally speaking, a compound is considered an antagonist of a given receptor subtype if in the below described co-tranfection assays the compound does not cause significant transcriptional activation of the receptor regulated reporter gene, but nevertheless binds to the receptor with a K_d value of less than approximately 1 μM.

Whether a compound is an RAR antagonist and therefore can be utilized in accordance with the present invention, can be tested in the following assays.

A chimeric receptor transactivation assay which tests for agonist-like activity in the RAR-α, RAR-β, RAR-γ, RXR-α receptor subtypes, and which is based on work published by Feigner P. L. and Holm M. *Focus* Vol 11, No. 2 (1989) is described in detail in published PCT Application No. WO94/17796, published on Aug. 18, 1994. The latter publication is the PCT counterpart of U.S. application Ser. No. 08/016,404, filed on Feb. 11, 1993, which issued as U.S. Pat. No. 5,455,265. PCT publication WO94/17796 and the specification of U.S. Pat. No. 5,455,265 are hereby expressly incorporated by reference. A compound should not cause significant activation of a reporter gene through a given receptor subtype (RAR-α, RAR-β or RAR-γ) in this assay, in order to qualify as an RAR antagonist with utility in the present invention.

A holoreceptor transactivation assay and a ligand binding assay which measure the antagonist/agonist like activity of the compounds of the invention, or their ability to bind to the several retinoid receptor subtypes, respectively, are described in published PCT Application No. WO93/11755 particularly on pages 30–33 and 37–41) published on Jun. 24, 1993, the specification of which is also incorporated herein by reference. A description of the holoreceptor transactivation assay is also provided below.

Holoreceptor Transactivation Assay

CV1 cells (5,000 cells/well) were transfected with an RAR reporter plasmid MTV-TREp-LUC (50 ng) along with one of the RAR expression vectors (10 ng) in an automated 96-well format by the calcium phosphate procedure of Heyman et al. *Cell* 68: 397–406. For RXR-α and RXR-γ transactivation assays, an RXR-responsive reporter plasmid CRBPII-tk-LUC (50 ng) along with the appropriate RXR expression vectors (10 ng) was used substantially as described by Heyman et al. above, and Allegretto et al. *J Biol. Chem.* 268: 26625–26633. For RXR-β transactivation assays, an RXR-responsive reporter plasmid CPRE-tk-LUC (50 mg) along with RXR-β expression vector (10 mg) was used as described in above. These reporters contain DRI elements from human CRBPII and certain DRI elements from promotor, respectively (see Mangelsdorf et al. *The Retinoids: Biology, Chemistry and Medicine*, pp. 319–349, Raven Press Ltd., New York and Heyman et al., cited above). A β-galactosidase (50 ng) expression vector was used as an internal control in the transfections to normalize for variations in transfection efficiency. The cells were transfected in triplicate for 6 hours, followed by incubation with retinoids for 36 hours, and the extracts were assayed for luciferase and β-galactosidase activities. The detailed experimental procedure for holoreceptor transactivations has been described in Heyman et al. above, and Allegretto et al. cited above. The results obtained in this assay are expressed in EC_{50} numbers, as they are also in the chimeric receptor transactivation assay. The Heyman et al. *Cell* 68:

397–406, Allegretto et al. *J. Biol. Chem.* 268: 26625–26633, and Mangelsdorf et al. *The Retinoids: Biology, Chemistry and Medicine,* pp. 319–349, Raven Press Ltd., New York, are expressly incorporated herein by reference. The results of ligand binding assay are expressed in K_d numbers. (See Cheng et al. *Biochemical Pharmacology* 22: 3099–3108, expressly incorporated herein by reference.)

A compound should not cause significant activation of a reporter gene through a given receptor subtype (RAR-α, RAR-β or RAR-γ) in the holoreceptor transactivation assay assay, in order to qualify as an RAR antagonist with utility in the present invention. Last, but not least, a compound should bind to at least one of the RAR receptor subtypes in the ligand binding assay with a K_d of less than approximately 1 micromolar (K_d<1 μM) in order to be capable of functioning as an antagonist of the bound receptor subtype, provided the same receptor subtype is not significantly activated by the compound.

Table 3 below shows the results of the holoreceptor transactivation assay and Table 4 discloses the efficacy (in percentage) in this assay of the test compound relative to all trans retinoic acid, for certain exemplary compounds of the invention. Table 5 shows the results of the ligand binding assay for certain exemplary compounds of the invention.

TABLE 3

Holoreceptor Transactivation Assay

Compound	EC_{50} (nanomolar)					
#	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
18	0.00	0.00	0.00	0.00	0.00	0.00
19	0.00	0.00	0.00	0.00	0.00	0.00
20	0.00	0.00	0.00	0.00	0.00	0.00
21	0.00	0.00	0.00	0.00	0.00	0.00
22	0.00	0.00	0.00	0.00	0.00	0.00
23	0.00	0.00	0.00	0.00	0.00	0.00
24	0.00	0.00	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.00	0.00	0.00
26	0.00	0.00	0.00	0.00	0.00	0.00
27	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00
34	0.00	0.00	0.00	0.00	0.00	0.00
36	0.00	0.00	0.00	0.00	0.00	0.00
39	0.00	0.00	0.00	0.00	0.00	0.00
41	0.00	0.00	0.00	0.00	0.00	0.00
45	0.00	0.00	0.00	0.00	0.00	0.00
46b	0.00	0.00	0.00	0.00	0.00	0.00
52	0.00	0.00	0.00	0.00	0.00	0.00
60	0.00	0.00	0.00	0.00	0.00	0.00
61	0.00	0.00	0.00	0.00	0.00	0.00
63	0.00	0.00	0.00	0.00	0.00	0.00
101	0.00	0.00	0.00	0.00	0.00	0.00
103	0.00	0.00	0.00	0.00	0.00	0.00

0.0 in Table 3 indicates that the compound is less than 20% as active (efficacious) in this assay than all trans retinoic acid.

TABLE 4

Transactivation Assay Efficacy (% of RA activity)

Compound #	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
18	4.00	1.00	0.00	0.00	10.00	1.0
19	0.00	5.0	3.0	0.0	9.0	4.0
20	3.0	4.0	0.00	4.00	0.00	3.0

TABLE 4-continued

Transactivation Assay Efficacy (% of RA activity)

Compound #	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
21	2.00	2.00	2.00	3.00	0.00	3.00
22	0.00	0.00	2.00	1.00	0.00	2.00
23	0.00	6.00	3.00	1.00	0.00	4.00
24	3.00	7.00	4.00	1.00	0.00	3.00
25	2.00	3.00	3.00	5.00	0.00	3.00
26	1.00	6.00	0.00	2.00	0.00	3.00
27	9.00	14.00	6.00	2.00	0.00	4.00
28	2.00	10.00	2.00	2.00	0.00	3.00
29	0.00	6.00	11.00	0.00	6.00	2.00
30	3.00	5.90	1.00	0.00	9.00	3.00
31	4.00	14.00	2.00	1.00	8.00	6.00
32	0.00	2.00	2.00	1.00	0.00	2.00
34	3.00	5.00	2.00	1.00	0.00	3.00
36	1.00	5.00	0.00	1.00	7.00	2.00
39	1.00	7.00	9.00	2.00	0.00	1.00
41	3.00	5.00	6.00	1.00	0.00	3.00
45	2.00	0.00	7.00	3.00	8.00	0.00
46b	4.00	5.00	3.00	2.00	0.00	4.00
52	0.00	15.00	3.00	0.00	0.00	10.00
60	0.00	1.00	4.00	3.00	0.00	3.00
61	2.00	2.00	0.00	1.00	0.00	3.00
63	2.00	2.00	7.00	1.00	0.00	1.00
101	0.00	4.00	2.00	1.00	0.00	3.0
103	4.00	12.0	7.0	0.00	0.0	2.0

TABLE 5

Ligand Binding Assay

Compound	K_d (nanomolar)					
#	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
18	24.00	11.00	24.00	0.00	0.00	0.00
19	565	210	659	0.00	0.00	0.00
20	130.00	22.0	34.00	0.00	0.00	0.00
21	16	9	13	0.00	0.00	0.00
22	24.0	17.0	27.0	0.00	0.00	0.00
23	32.00	25.00	31.00	0.00	0.00	0.00
24	699	235	286	0.00	0.00	0.00
25	50	17	20	0.00	0.00	0.00
26	40.00	31.00	36.00	0.00	0.00	0.00
27	69.00	14.00	26.00	0.00	0.00	0.00
28	669	77	236	0.00	0.00	0.00
29	234	48	80	0.00	0.00	0.00
30	683	141	219	0.00	0.00	0.00
31	370	52.00	100.00	0.00	0.00	0.00
32	0.00	89.00	169.00	0.00	0.00	0.00
34	52.00	30.00	17.00	0.00	0.00	0.00
36	13.00	550.00	0.00	0.00	0.00	0.00
39	67.00	38.00	113.00	0.00	0.00	0.00
41	5.1	491	725	0.00	0.00	0.00
45	12.0	2.80	17.0	0.00	0.00	0.00
46b	250	3.70	5.80	0.00	0.00	0.00
52	60.00	63.00	5.6.00	0.00	0.00	0.00
60	1.5	1.9	3.3	0.00	0.00	0.00
61	96	15	16	0.00	0.00	0.00
63	133	3219	0.00	0.00	0.00	0.00
101	750	143	637	0.00	0.00	0.00
103	301	273	261	0.00	0.00	0.00

0.0 in Table 5 indicates a value greater than 1000 nM.

As it can be seen from the test results summarized in Tables 3, 4 and 5, the therein indicated exemplary compounds of the invention are antagonists of the RAR receptor subtypes, but have no affinity to RXR receptor subtypes. (Other compounds of the invention may be antagonist of some but not all RAR receptor subtypes and agonists of the remaining RAR subtypes.) Due to this property, the compounds of the invention can be used to block the activity of

RAR agonists in biological assays. In mammals, including humans, the compounds of the invention can be coadministered with RAR agonists and, by means of pharmacological selectivity or site-specific delivery, preferentially prevent the undesired effects of RAR agonists. The compounds of the invention can also be used to treat Vitamin A overdose, acute or chronic, resulting either from the excessive intake of vitamin A supplements or from the ingestion of liver of certain fish and animals that contain high level of Vitamin A. Still further, the compounds of the invention can also be used to treat acute or chronic toxicity caused by retinoid drugs. It has been known in the art that the toxicities observed with hypervitamninosis A syndrome (headache, skin peeling, bone toxicity, dyslipidemias) are similar or identical with toxicities observed with other retinoids, suggesting a common biological cause, that is RAR activation. Because the compounds of the present invention block RAR activation, they are suitable for treating the foregoing toxicities.

The compounds of the invention are able to substantially prevent skin irritation induced by RAR agonist retinoids, when the compound of the invention is topically coadministered to the skin. Similarly, compounds of the invention can be administered topically to the skin, to block skin irritation, in patients or animals who are administered RAR agonist compounds systemically. The compounds of the invention can accelerate recovery from pre-existing retinoid toxicity, can block hypertriglyceridemia caused by co-administered retinoids, and can block bone toxicity induced by an RAR agonist (retinoid).

Generally speaking, for therapeutic applications in mammals in accordance with the present invention, the antagonist compounds can be admistered enterally or topically as an antidote to vitamin A, vitamin A precursors, or antidote to retinoid toxicity resulting from overdose or prolonged exposure, after intake of the causative factor (vitamin A precursor or other retinoid) has been discontinued. Alternatively, the antagonist compounds are coadministered with retinoid drugs in accordance with the invention, in situations where the retinoid provides a therapeutic benefit, and where the coadministered antagonist alleviates or eliminates one or more undesired side effects of the retinoid. For this type of application the antagonist may be administered in a site-specific manner, for example as a topically applied cream or lotion while the coadministered retinoid may be given enterally.

For therapeutic applications in accordance with the present invention the antagonist compounds are incorporated into pharmaceutical compositions, such as tablets, pills, capsules, solutions, suspensions, creams, ointments, gels, salves, lotions and the like, using such pharmaceutically acceptable excipients and vehicles which per se are well known in the art. For example preparation of topical formulations are well described in *Remington's Pharmaceutical Science,* Edition 17, Mack Publishing Company, Easton, Pa. For topical application, the antagonist compounds could also be administered as a powder or spray, particularly in aerosol form. If the drug is to be administered systemically, it may be confected as a powder, pill, tablet or the like or as a syrup or elixir suitable for oral administration. For intravenous or intraperitoneal administration, the antagonist compound will be prepared as a solution or suspension capable of being administered by injection. In certain cases, it may be useful to formulate the antagonist compounds by injection. In certain cases, it may be useful to formulate the antagonist compounds in suppository form or as extended release formulation for deposit under the skin or intramuscular injection.

The antagonist compounds will be administered in a therapeutically effective dose in accordance with the invention. A therapeutic concentration will be that concentration which effects reduction of the particular condition (such as toxicity due to retinoid or vitamin A exposure, or side effect of retinoid drug) or retards its expansion. It should be understood that when coadministering the antagonist compounds to block retinoid-induced toxicity or side effects in accordance with the invention, the antagonist compounds are used in a prophylactic manner to prevent onset of a particular condition, such as skin irritation.

A useful therapeutic or prophylactic concentration will vary from condition to condition and in certain instances may vary with the severity of the condition being treated and the patient's susceptibility to treatment. Accordingly, no single concentration will be uniformly useful, but will require modification depending on the particularities of the chronic or acute retinoid toxicity or related condition being treated. Such concentrations can be arrived at through routine experimentation. However, it is anticipated that a formulation containing between 0.01 and 1.0 milligrams of antagonist compound per mililiter of formulation will constitute a therapeutically effective concentration for topical application. If administered systemically, an amount between 0.01 and 5 mg per kg per day of body weight would be expected to effect a therapeutic result.

The basis of the utility of RAR antagonists for the prevention or treatment of RAR agonist-induced toxicity is competitive inhibition of the activation of RAR receptors by RAR agonists. The main distinction between these two applications of RAR antagonists is the presence or absence of preexisting retinoid toxicity. Most of the examples immediately described below relate to the use of retinoids to prevent retinoid toxicity, but the general methods described herein are applicable to the treatment of preexisting retinoid toxicity as well.

Description of Experiments Demonstrating the Use of RAR Antagonists to Prevent or Treat Retinoid Toxicity and/or Side Effects of Retinoid Drugs

EXAMPLE 1

Skin Iritation Induced by Topically Applied Agonist is Treated with Topically Applied Antagonist

The compound 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)propen-1-yl]benzoic acid, designated AGN 191183, is known in the prior art as a potent RAR agonist (see for example the descriptive portion and FIG. 2*b* of U.S. Pat. No. 5,324,840). (The "AGN" number is an arbitrarily designated reference number utilized by the corporate assignee of the present invention for identification of compounds.)

4-[(5,6-dihydro-5,5-dimethyi-8-(phenyl)-2-naphthalenyl) ethynyl]benzoic acid (AGN 192869, also designated Compound 60a) is a compound the preparation of which is described below. This compound is an RAR antagonist.

Skin irritation induced by an RAR agonist, AGN 191183, administered topically, can be blocked by an RAR antagonist, AGN 192869, also administered topically in hairless mice.

More particularly skin irritation was measured on a semi-quantitative scale by the daily subjective evaluation of skin flaking and abrasions. A single number, the topical irritation score, summarizes the skin irritation induced in an animal during the course of an experiment. The topical irritation

score is calculated as follows. The topical irritation score is the algebraic sum of a composite flaking score and a composite abrasion score. The composite scores range from 0–9 and 0–8 for flaking and abrasions, respectively, and take into account the maximum severity, the time of onset, and the average severity of the flaking and abrasions observed.

The severity of flaking is scored on a 5-point scale and the severity of abrasions is scored on a 4-point scale, with higher scores reflecting greater severity. The maximum severity component of the composite scores would be the highest daily severity score assigned to a given animal during the course of observation.

For the time of onset component of the composite score, a score ranging from 0 to 4 is assigned as follows:

TABLE 6

Time to Appearance of Flaking or Abrasions of Severity 2 or Greater	
(days)	Time of Onset Score
8	0
6–7	1
5	2
3–4	3
1–2	4

The average severity component of the composite score is the sum of the daily flaking or abrasion scores divided by the number of observation days. The first day of treatment is not counted, since the drug compound has not had an opportunity to take effect at the time of first treatment.

To calculate the composite flaking and abrasion scores, the average severity and time of onset scores are summed and divided by 2. The result is added to the maximal severity score. The composite flaking and abrasion scores are then summed to give the overall topical irritation score. Each animal receives a topical irritation score, and the values are expressed as the mean ±SD of the individual scores of a group of animals. Values are rounded to the nearest integer.

Female hairless mice [Crl:SKH1-hrBR] (8–12 weeks old, n=6) were treated topically for 5 consecutive days with acetone, AGN 191183, AGN 192869, or some combination of AGN 192869 and 191183. Doses of the respective compounds are given in Table 7. Treatments are applied to the dorsal skin in a total volume of 4 ml/kg (~0.1 ml). Mice were observed daily and scored for flaking and abrasions up to and including 3 days after the last treatment, i.e., day 8.

TABLE 7

Experimental Design and Results Example 1

Group	Dose AGN 191183 (mg/kg/d)	Dose AGN 192869 (mg/kg/d)	Molar Ratio (192869: (191183	Topical Irritation Score)
A	0	0	—	0 ± 0
B	0.025	0	—	8 ± 2
C	0.025	0.06	2:1	5 ± 2
D	0.025	0.30	10:1	2 ± 1
E	0.025	1.5	50:1	1 ± 0
F	0	1.5	—	0 ± 0

The topical irritation scores for Example 1 are given in Table 7. Neither acetone (vehicle) nor AGN 192869 (antagonist) at a dose of 1.5 mg/kg/d (group F) caused observable topical irritation. AGN 191183, the RAR agonist, caused modest topical irritation at a dose of 0.025 mg/kg/d. However, AGN 191183-induced topical irritation was inhibited in a dose-dependent fashion by AGN 192869, with nearly complete abrogation of irritation in the presence of a 50-fold molar excess of AGN 192869. This demonstrates that a topical RAR antagonist blocks skin irritation caused by a topical RAR agonist. Complete blockade of RAR agonist-induced skin irritation can be achieved with lower molar ratios of antagonist to agonist when the RAR antagonists is more potent, such as the compound 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl] benzoic acid (AGN 193109, also designated in this application as Compound 60.)

EXAMPLE 2

Skin Iritation Induced by Orally Applied Agonist is Blocked with Topically Applied Antagonist

The potent RAR agonist AGN 191183 (4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)propen-1-yl]benzoic acid) and the potent RAR antagonist 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl) ethynyl]benzoic acid (AGN 193109, Compound 60) were used in this example and body weight of the experimental animals (mice) was used as a marker of systemic RAR agonist exposure.

Groups of female hairless mice (8–12 weeks old, n=6) were treated by intragastric intubation with corn oil or AGN 191183 (0.26 mg/kg) suspended in corn oil (5 ml/kg). Mice were simultaneously treated topically on the dorsal skin with vehicle (97.6% acetone/2.4% dimethylsulfoxide) or solutions of AGN 193109 in vehicle (6 ml/kg). Specific doses for the different treatment groups are give in Table 8. Treatments were administered daily for 4 consecutive days. Mice were weighed and graded for topical irritation daily as described in Example 1 up to and including 1 day after the last treatment. Percent body weight change is calculated by subtracting final body weight (day 5) from initial body weight (day 1), dividing by initial body weight, and multiplying by 100%. Topical irritation scores are calculated as described in Example 1.

Topical irritation scores and weight loss for the different groups are given in Table 8. Combined treatment with the topical and oral vehicles, i.e., acetone and corn oil, respectively, caused no topical irritation or weight loss. Similarly, combined treatment with the oral vehicle and the topical antagonist AGN 193109 resulted in no topical irritation or weight loss. Oral AGN 191183 by itself induced substantial weight loss and skin irritation. AGN 191183-induced skin irritation was substantially reduced when combined with the lower dose of AGN 193109 and completely blocked at the higher dose of AGN 193109. AGN 191183-induced weight loss was also blocked in a dose-related fashion by topical AGN 193109, but the blockade was not complete. Thus, topical AGN 193109 preferentially blocked the dermal toxicity of AGN 191183. Presumably, low levels of AGN 193109 were absorbed systemically and thus partially blocked the weight loss induced by AGN 191183. However, such absorption would likely be even less in a species with less permeable skin, such as humans. Alternatively, the weight loss inhibition by AGN 193109 could be due to amelioration of the AGN 191183 induced skin irritation.

TABLE 8

Experimental Design and Results Example 2

Group	Dose of Topical AGN 193109 (mg/kg/d)	Dose of Oral AGN 191183 (mg/kg/d)	% Weight Gain or (Loss)	Topical Irritation Score)
A	0	0	1 ± 2	0 ± 0
B	0	0.26	(21 ± 6)	8 ± 1
C	0.12	0.26	(9 ± 5)	1 ± 1
D	0.47	0.26	(3 ± 5)	0 ± 1
E	0.47	0	3 ± 3	0 ± 0

Thus, Example 2 demonstrates that RAR antagonists administered topically can be used to block preferentially the skin irritation induced by an RAR agonist administered orally.

EXAMPLE 3

Topically Applied Antagonist Accelarates Recovery from Prexisting Retinoid Toxicity

In this example, weight loss is induced by topical treatment with the RAR agonist AGN 191183 and then the test animals are topically treated with either vehicle or the RAR antagonist AGN 193109.

Female hairless mice (8–12 weeks old, n=5) were treated topically with AGN 191183 (0.13 mg/kg/d) in vehicle (97.6% acetone/2.4% DMSO, 4 ml/kg) daily for 2 days. Groups of these same mice (n=5) were then treated topically either with vehicle or AGN 193109 in vehicle (4 ml/kg) daily for 3 consecutive days beginning on day 3. Mice were weighed on days 1–5 and on day 8. Body weights are expressed as the mean ±SD. Means were compared statistically using an unpaired, two-tailed t-test. Differences were considered significant at $P<0.05$.

TABLE 9

Results Example 3

Treatment (days 3–5)	Body Weight (g) DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 8
vehicle	24.6 ± 1.5	23.9 ± 12	21.4 ± 1.2	20.3 ± 1.7	21.0 ± 1.4	24.7 ± 1.0
AGN 193109	23.9 ± 1.0	23.5 ± 1.2	21.4 ± 0.6	22.2 ± 0.7	22.8 ± 0.8	25.0 ± 1.1

The time course of body weights in Example 3 are given in Table 9. Body weights of both groups of mice were lowered in parallel on days 2 and 3 as a result of AGN 191183 treatment on days 1 and 2. Body weights in the two groups were not significantly different on days 1, 2, or 3. However, AGN 193109 treatment significantly increased body weight relative to vehicle treatment on days 4 and 5. These data indicated that recovery from AGN 191183-induced body weight loss was accelerated by subsequent treatment with AGN 193109. Body weights were not significantly different between the two groups of mice on day 8, indicating that full recovery was achievable in both groups given sufficient time. Thus, RAR antagonists are effective in alleviating RAR agonist-induced toxicity even if RAR agonist-induced toxicity precedes RAR antagonist treatment, i.e., in the RAR agonist poisoning scenario.

EXAMPLE 4

Orally Applied Antagonist Blocks Hypertriglyceridemia Incuded by Orally Coadministered Retinoid Agonist

5-[(E)-2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalen-2-yl)propen-1-yl]-2-thiophencarboxylic acid, is a known RAR/RXR pan-agonist (see U.S. Pat. No. 5,324,840 column 32) and is designated AGN 191659. This compound was used orally to induce acute hypertriglyceridemia in rats, and AGN 193109 Compound 60 was coadministered orally to block the AGN 191659-induced hypertriglyceridemia.

Male Fischer rats (6–7 weeks old, n=5) were treated by intragastric intubation with corn oil (vehicle), AGN 191659, AGN 193109 or a combination of AGN 191659 and AGN 193109. AGN 191659 and AGN 193109 were given as fine suspensions in corn oil. The experimental design, including doses, is given in Table 10.

Blood was withdrawn from the inferior vena cava under carbon dioxide narcosis. Serum was separated from blood by low speed centrifugation. Total serum triglycerides (triglycerides plus glycerol) were measured with a standard spectrophotometric endpoint assay available commercially as a kit and adapted to a 96-well plate format. Serum triglyceride levels are expressed as the mean ±SD. Means were compared statistically by one-way analysis of variance followed by Dunnett's test if significant differences were found. Differences were considered significant at $P<0.05$.

As shown in Table 10, AGN 191659 by itself caused significant elevation of serum triglycerides relative to vehicle treatment. AGN 193109 by itself did not significantly increase serum triglycerides. Importantly, the combination of AGN 193109 and AGN 191659 at molar ratios of 1:1 and 5:1 reduced serum triglycerides to levels that were not signficantly different from control.

TABLE 10

Experimental Design and Results Example 4

Group	Treatment (dose)	Serum Triglycerides (mg/dl)
A	vehicle	55.0 ± 3.1
B	AGN 193109 (19.6 mg/kg)	52.4 ± 6.3
C	AGN 191659 (3.7 mg/kg)	122.5 ± 27.6
D	AGN 193109 (3.9 mg/kg) + AGN 191659 (3.7 mg/kg)	55.7 ± 14.7
E	AGN 193109 (19.6 mg/kg) + AGN 191659 (3.7 mg/kg)	72.7 ± 8.9

Example 4 demonstrates that an RAR antagonist can be used to block hypertriglyceridemia induced by a coadministered retinoid.

EXAMPLE 5

Parenterally Applied Antagonist Blocks Bone Toxicity Incuded by Parenterally Coadministered Retinoid Agonist

Example 5 demonstrates that RAR antagonists can block bone toxicity induced by an RAR agonist. In this example, AGN 193109 is used to block premature epiphyseal plate closure caused by a coadministered RAR agonist, AGN 191183, in guinea pigs.

Groups of male Hartley guinea pigs (~3 weeks old, n=4) were implanted intraperitoneally with osmotic pumps containing vehicle (20% dimethylsulfoxide/80% polyethylene glycol-300), AGN 191183 (0.06 mg/ml), or AGN 191183 (0.06 mg/ml) in combination with AGN 193109 (0.34 mg/ml). The osmotic pumps are designed by the manufacturer to deliver ~5 μl of solution per hour continuously for 14 days.

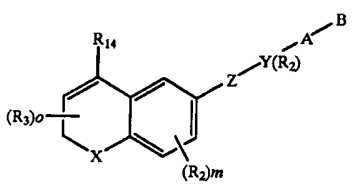
The animals were euthanized by carbon dioxide asphyxiation 14 days after implantation. The left tibia was was removed and placed in 10% buffered formalin. The tibias were decalcified by exposure to a formic acid/formalin solution for 3–4 days, and paraffin sections were prepared. Sections were stained with hematoxylin and eosin by standard methods. The proximal tibial epiphyseal plate was examined and scored as closed or not closed. Epiphyseal plate closure is defined for this purpose as any interruption of the continuity of the epiphyseal growth plate cartilage, i.e., replacement by bone and/or fibroblastic tissue.

None of the four vehicle-treated guinea pigs showed epiphyseal plate closure by the end of the experiment. This was expected, since the proximal epiphyseal plate of guinea pig tibia does not normally close until the animals are at least 10 months old. All four of the AGN 191183-treated guinea pigs showed partial or complete epiphyseal plate closure. However, none of the guinea pigs treated with the combination of AGN 191183 and AGN 193109 demonstrated epiphyseal plate closure. Thus, AGN 193109 at a 5-fold molar excess completely blocked AGN 191183-induced bone toxicity when these compounds were coadministered parenterally.

RAR Antagonist Compounds

The compounds 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (AGN 193109, Compound 60) and 4-[(5,6-dihydro-5,5-dimethyl-8-(phenyl)-2-naphthalenyl)ethynyl]benzoic acid (AGN 192869, Compound 60a) are examples of RAR antagonists which were used in the above-described animal tests for blocking RAR receptors in accordance with the present invention. The compounds of the following formula (Formula 1) serve as further and general examples for additional RAR antagonist compounds for use in accordance with the present invention.

Formula 1



In Formula 1, X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons, or

X is $[C(R_1)_2]_n$ where R_1 is H or alkyl of 1 to 6 carbons, and n is an integer between 0 or 1;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, CF_3, fluor substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C≡C—,

—N=N—,

—N=CR_1—,

—CR_1=N,

—$(CR_1=CR_1)_{n'}$— where n' is an integer having the value 0–5,

—CO—NR_1—,

—CS—NR_1—,

—NR_1—CO,

—NR_1—CS,

—COO—,

—OCO—;

—CSO—;

—OCS—;

Y is a phenyl or naphthyl group, or heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said phenyl and heteroaryl groups being optionally substituted with one or two R_2 groups, or when Z is $—(CR_1=CR_1)_{n'}—$ and n' is 3, 4 or 5 then Y represents a direct valence bond between said $(CR_2=CR_2)_{n'}$ group and B;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

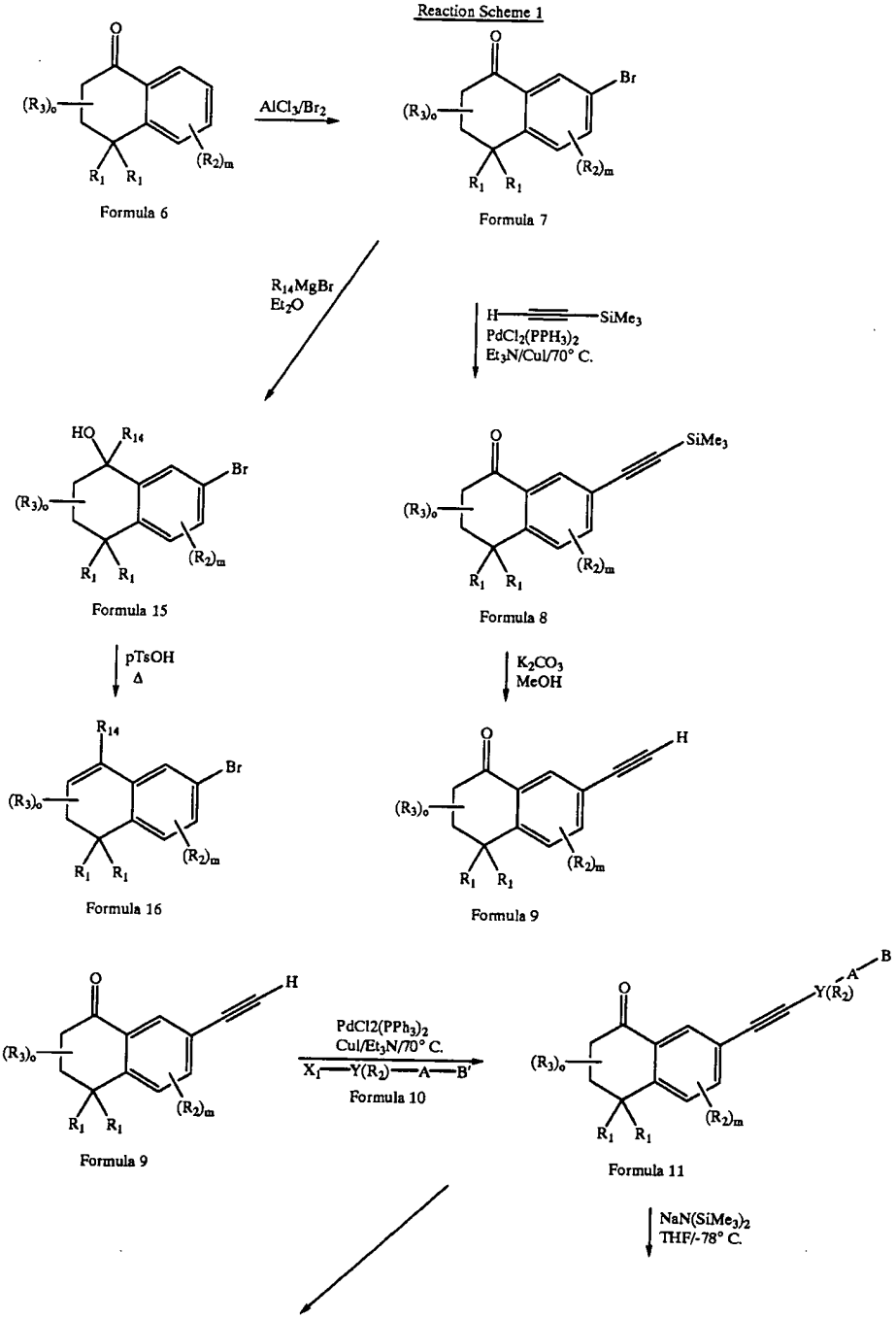
R_{14} is $(R_{15})_r$-phenyl, $(R_{15})_r$-naphthyl, or $(R_{15})_r$-heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $N(R_8)COR_8$, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

Synthetic Methods—Aryl Substituted Compounds

The exemplary RAR antagonist compounds of Formula 1 can be made by the synthetic chemical pathways illustrated here. The synthetic chemist will readily appreciate that the conditions set out here are specific embodiments which can be generalized to any and all of the compounds represented by Formula 1.

Reaction Scheme 1



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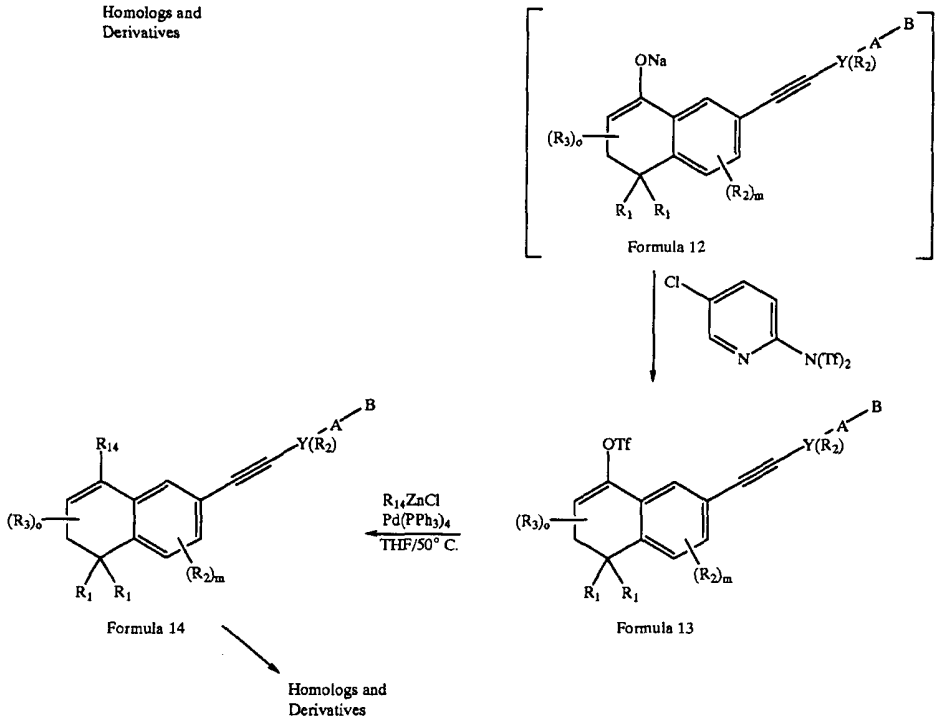


Reaction Scheme 1 illustrates the synthesis of compounds of Formula 1 where the Z group is an ethynyl function (—C≡C—) and X is $[C(R_1)_2]_n$ where n is 1. In other words, Reaction Scheme 1 illustrates the synthesis of ethynyl substituted dihydronaphthalene derivatives of the present invention. In accordance with this scheme, a tetrahydronaphtalene-1-one compound of Formula 6 is brominated to provide the bromo derivative of Formula 7. The compounds of Formula 6 already carry the desired R_1, R_2 and R_3 substituents, as these are defined above in connection with Formula 1. A preferred example of a compound of Formula 6 is 3,4-dihydro-4,4-dimethyl-1(2H)-naphthalenone, which is described in the chemical literature (Arnold et al. *J. Am. Chem. Soc.* 69: 2322–2325 (1947)). A presently preferred route for the synthesis of this compound from 1-bromo-3-phenylpropane is also described in the experimental section of the present application.

The compounds of Formula 7 are then reacted with (trimethylsilyl)acetylene to provide the (trimethylsilyl) ethynyl-substituted 3,4-dihydro-naphthalen-1(2H)-one compounds of Formula 8. The reaction with (trimethylsilyl) acetylene is typically conducted under heat (approximately 100° C.) in the presence of cuprous iodide, a suitable catalyst, typically having the formula $Pd(PPh_3)_2Cl_2$, an acid acceptor (such as triethylamine) under an inert gas (argon) atmosphere. Typical reaction time is approximately 24 hours. The (trimethylsilyl)ethynyl-substituted 3,4-dihydro-naphthalen-1(2H)-one compounds of Formula 8 are then reacted with base (potassium hydroxide or potassium carbonate) in an alcoholic solvent, such as methanol, to provide the ethynyl substituted 3,4-dihydro-1-naphthalen-1(2H)ones of Formula 9. Compounds of Formula 9 are then coupled with the aromatic or heteroaromatic reagent X_1-Y(R_2)-A-B' (Formula 10) in the presence of cuprous iodide, a suitable catalyst, typically $Pd(PPh_3)_2Cl_2$, an acid acceptor, such as triethylamine, under inert gas (argon) atmosphere. Alternatively, a zinc salt (or other suitable metal salt) of the compounds of Formula 9 can be coupled with the reagents of Formula 10 in the presence of $Pd(PPh_3)_4$ or similar complex. Typically, the coupling reaction with the reagent X_1-Y(R_2)-A-B' (Formula 10) is conducted at room or moderately elevated temperature. Generally speaking, coupling between an ethynylaryl derivative or its zinc salt and a halogen substituted aryl or heteroaryl compound, such as the reagent of Formula 10, is described in U.S. Pat. No. 5,264,456, the specification of which is expressly incorporated herein by reference. The compounds of Formula 11 are precursors to exemplary compounds of the present invention, or derivatives thereof protected in the B' group, from which the protecting group can be readily removed by reactions well known in the art. The compounds of Formula 11 can also be converted into further precursors to the exemplary compounds by such reactions and transformations which are well known in the art. Such reactions are indicated in Reaction Scheme 1 by conversion into "homologs and derivatives". One such conversion employed for the synthesis of several exemplary compounds is saponification of an ester group (when B or B' is an ester) to provide the free carboxylic acid or its salt.

The halogen substituted aryl or heteroaryl compounds of Formula 10 can, generally speaking, be obtained by reactions well known in the art. An example of such compound is ethyl 4-iodobenzoate which is obtainable, for example, by esterification of 4-iodobenzoic acid. Another example is ethyl 6-iodonicotinoate which can be obtained by conducting a halogen exchange reaction on 6-chloronicotinic acid, followed by esterification. Even more generally speaking, regarding derivatization of compounds of Formula 11 and/or

the synthesis of aryl and heteroaryl compounds of Formula 10 which can thereafter be reacted with compounds of Formula 9, the following well known and published general principles and synthetic methodology can be employed.

Carboxylic acids are typically esterified by refluxing the acid in a solution of the appropriate alcohol in the presence of an acid catalyst such as hydrogen chloride or thionyl chloride. Alternatively, the carboxylic acid can be condensed with the appropriate alcohol in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine. The ester is recovered and purified by conventional means. Acetals and ketals are readily made by the method described in March, *Advanced Organic Chemistry,* 2nd Edition, McGraw-Hill Book Company, p. 810). Alcohols, aldehydes and ketones all may be protected by forming respectively, ethers and esters, acetals or ketals by known methods such as those described in McOmie, Plenum Publishing Press, 1973 and Protecting Groups, Ed. Greene, John Wiley & Sons, 1981.

To increase the value of n in the compounds of Formula 10 before affecting the coupling reaction of Reaction Scheme 1 (where such compounds corresponding to Formula 10 are not available from a commercial source) aromatic or heteroaromatic carboxylic acids are subjected to homologation by successive treatment under Arndt-Eistert conditions or other homologation procedures. Alternatively, derivatives which are not carboxylic acids may also be homologated by appropriate procedures. The homologated acids can then be esterified by the general procedure outlined in the preceding paragraph.

Compounds of Formula 10, (or other intermediates or exemplary compounds) where A is an alkenyl group having one or more double bonds can be made for example, by synthetic schemes well known to the practicing organic chemist; for example by Wittig and like reactions, or by introduction of a double bond by elimination of halogen from an alpha-halo-arylalkyl-carboxylic acid, ester or like carboxaldehyde. Compounds of Formula 10 (or other intermediates or exemplary compounds) where the A group has a triple (acetylenic) bond can be made by reaction of a corresponding aromatic methyl ketone with strong base, such as lithium diisopropylamide, reaction with diethyl chlorophosphate and subsequent addition of lithium diisopropylamide.

The acids and salts derived from compounds of Formula 11 (or other intermediates or exemplary compounds) are readily obtainable from the corresponding esters. Basic saponification with an alkali metal base will provide the acid. For example, an ester of Formula 11 (or other intermediates or exemplary compounds) may be dissolved in a polar solvent such as an alkanol, preferably under an inert atmosphere at room temperature, with about a three molar excess of base, for example, lithium hydroxide or potassium hydroxide. The solution is stirred for an extended period of time, between 15 and 20 hours, cooled, acidified and the hydrolysate recovered by conventional means.

The amide may be formed by any appropriate amidation means known in the art from the corresponding esters or carboxylic acids. One way to prepare such compounds is to convert an acid to an acid chloride and then treat that compound with ammonium hydroxide or an appropriate amine.

Alcohols are made by converting the corresponding acids to the acid chloride with thionyl chloride or other means (J. March, *Advanced Organic Chemistry,* 2nd Edition, McGraw-Hill Book Company), then reducing the acid chloride with sodium borohydride (March, Ibid, p. 1124), which gives the corresponding alcohols. Alternatively, esters may be reduced with lithium aluminum hydride at reduced temperatures. Alkylating these alcohols with appropriate alky halides under Williamson reaction conditions (March, Ibid, p. 357) gives the corresponding ethers. These alcohols can be converted to esters by reacting them with appropriate acids in the presence of acid catalysts or dicyclohexylcarbodiimide and dimethylaminopyridine.

Aldehydes can be prepared from the corresponding primary alcohols using mild oxidizing agents such as pyridinium dichromate in methylene chloride (Corey, E. J., Schmidt, G., *Tet. Lett.* 399, 1979), or dimethyl sulfoxide/oxalyl chloride in methylene chloride (Omura, K., Swern, D., *Tetrahedron* 34: 1651 (1978)).

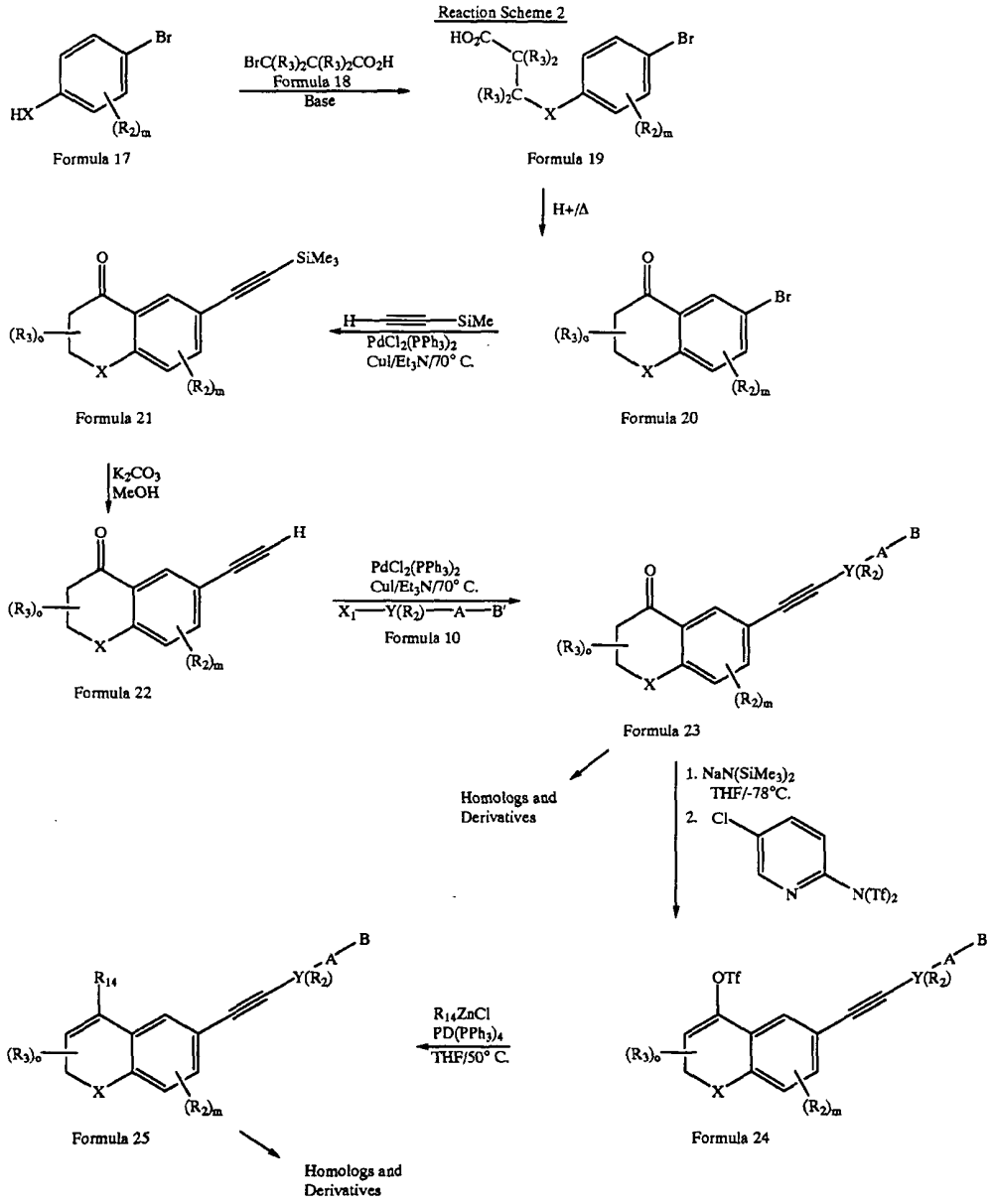
Ketones can be prepared from an appropriate aldehyde by treating the aldehyde with an alkyl Grignard reagent or similar reagent followed by oxidation.

Acetals or ketals can be prepared from the corresponding aldehyde or ketone by the method described in March, Ibid, p. 810.

Compounds of Formula 10 (or other intermediates, or exemplary compounds) where B is H can be prepared from the corresponding halogenated aromatic or hetero aromatic compounds, preferably where the halogen is I.

Referring back again to Reaction Scheme 1, the compounds of Formula 11 are reacted with sodium bis (trimethylsilyl)amide and 2-[N,N-bis (trifluoromethylsulfonyl)amino]-5-chloropyridine in an inert ether type solvent, such as tetrahydrofuran, at low temperatures (−78° C. and 0° C.). This is shown in Reaction Scheme 1 where the usually unisolated sodium salt intermediate is shown in brackets as Formula 12. The reaction results in the trifluoromethylsulfonyloxy derivatives represented in Formula 13. (Tf=SO_2CF_3). The compounds of Formula 13 are then converted to the exemplary compounds of the invention, shown in Formula 14, by reaction with an organometal derivative derived from the aryl or heteroaryl compound $R_{14}H$, such that the formula of the organometal derivative is R_{14}Met (Met stands for monovalent metal), preferably R_{14}Li. (R_{14} is defined as in connection with Formula 1.) The reaction with the organometal derivative, preferably lithium derivative of the formula R_{14}Li is usually conducted in an inert ether type solvent (such as tetrahydrofuran) in the presence of zinc chloride ($ZnCl_2$) and tetrakis(triphenylphosphine)-palladium(0) ($Pd(PPh_3)_4$). The organolithium reagent R_{14}Li, if not commercially available, can be prepared from the compound $R_{14}H$ (or its halogen derivative R_{14}-X_1 where X_1 is halogen) in an ether type solvent in accordance with known practice in the art. The temperature range for the reaction between the reagent R_{14}Li and the compounds of Formula 13 is, generally speaking in the range of approximately −78° C. to 50° C. The compounds of Formula 14 can be converted into further homologs and derivatives in accordance with the reactions discussed above.

The intermediate 7-bromo-tetrahydronaphthalene-1-one compounds of Formula 7 shown in Reaction Scheme 1 can also be converted with a Grignard reagent of the formula $R_{14}MgBr$ (R_{14} is defined as in connection with Formula 1) to yield the tertiary alcohol of Formula 15. The tertiary alcohol is dehydrated by treatment with acid to provide the 3,4-dihydro-7-bromonaphthalene derivatives of Formula 16, which serve as intermediates for the synthesis of additional compounds of the present invention (see Reaction Schemes 6, 7, and 8).



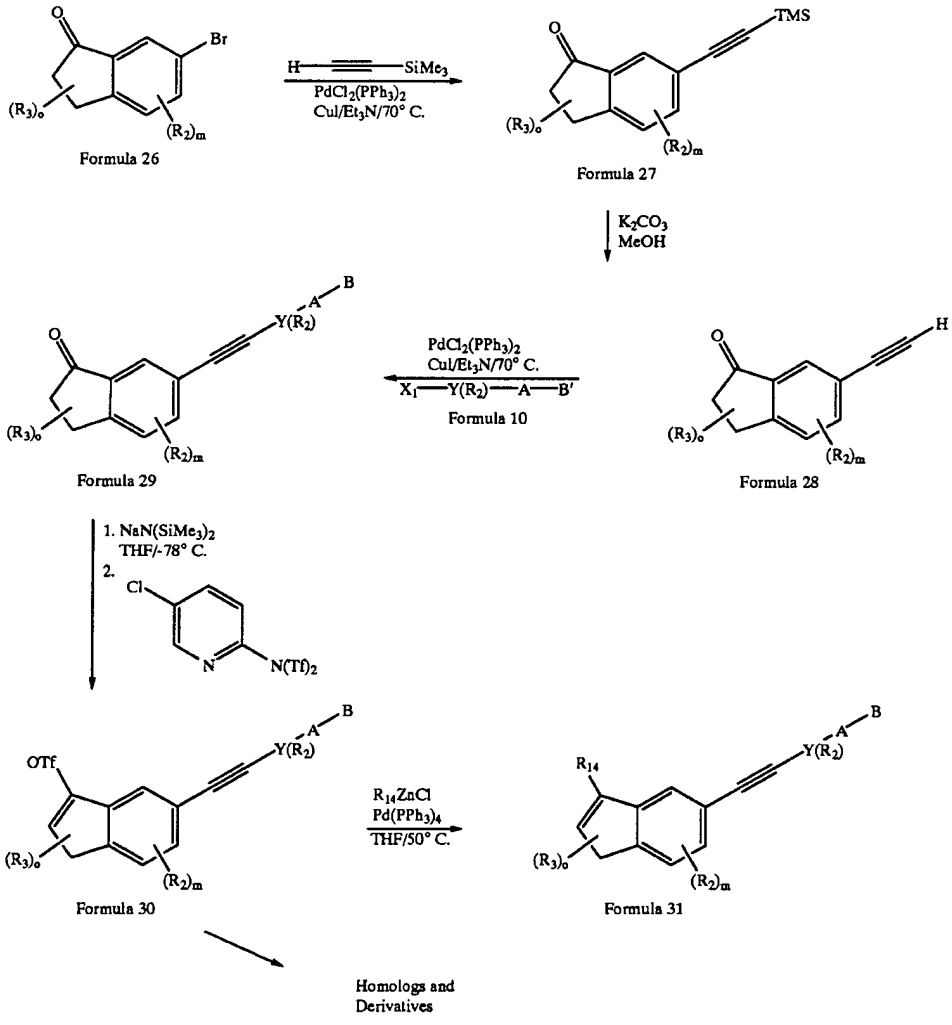
Referring now to Reaction Scheme 2 a synthetic route to those compounds is disclosed where with reference to Formula 1 X is S, O or NR' and the Z group is an ethynyl function (—C≡C—). Starting material for this sequence of the reaction is a bromophenol, bromothiophenol or bromoaniline of the structure shown in Formula 17. For the sake of simplifying the present specification, in the ensuing description X can be considered primarily sulfur as for the preparation of benzothiopyran derivatives. It should be kept in mind, however, that the herein described scheme is also suitable, with such modifications which will be readily apparent to those skilled in the art, for the preparation of benzopyran (X=O) and dihydroquinoline (X=NR') compounds of the present invention. Thus, the compound of Formula 17, preferably para bromothiophenol, para bromophenol or para bromoaniline is reacted under basic condition with a 3-bromo carboxylic acid of the Formula 18. In this reaction scheme the symbols have the meaning described in connection with Formula 1. An example for the reagent of Formula 18 where R_3 is hydrogen, is 3-bromopropionic acid. The reaction with the 3-bromocarboxylic acid of Formula 18 results in the compound of Formula 19. The latter is cyclized by treatment with acid to yield the 6-bromothiochroman-4-one derivative (when X is S) or 6-bromochroman derivative (when X is 0) of Formula 20. The bromo compounds of Formula 20 are then subjected to substantially the same sequence of reactions under analogous conditions, which are described in

Reaction Scheme 1 for the conversion of the bromo compounds of Formula 7 to the compounds of the invention. Thus, briefly summarized here, the bromo compounds of Formula 20 are reacted with (trimethylsilyl)acetylene to provide the 6-(trimethylsilyl)ethynyl-substituted thiochroman-4-one or chroman-4-one compounds of Formula 21. The 6-(trimethylsilyl)ethynyl-substituted thiochroman-4-one compounds of Formula 21 are then reacted with base (potassium hydroxide or potassium carbonate) to provide the ethynyl substituted 6-ethynyl substituted thiochroman-4-ones of Formula 22. Compounds of Formula 22 are then coupled with the aromatic or heteroaromatic reagent X_1-$Y(R_2)$-A-B' (Formula 10) under conditions analogous to those described for the analogous reactions of Reaction Scheme 1, to yield the compounds of Formula 23.

The compounds of Formula 23 are then reacted still under conditions analogous to the similar reactions described in Reaction Scheme 1 with sodium bis(trimethylsilyl)amide and 2-[N,N-bis(trifluoromethylsulfonyl)amino]-5-chloropyridine to yield the 4-trifluoromethylsulfonyloxy benzothiopyran or benzopyran derivatives represented in Formula 24. The compounds of Formula 24 are then converted to compounds shown in Formula 25, by reaction with an organometal derivative derived from the aryl or heteroaryl compound $R_{14}H$, as described in connection with Reaction Scheme 1.

Similarly to the use of the intermediate 7-bromotetrahydronaphthalene-1-one compounds of Formula 7 of Reaction Scheme 1, the intermediate 6-bromothiochroman-4-one compounds of Formula 20 can also be used for the preparation of further compounds within the scope of the present invention, as described below, in Reaction Schemes 6, 7 and 8. The compounds of Formula 25, can also be converted into further homologs and derivatives, in reactions analogous to those described in connection with Reaction Scheme 1.

Reaction Scheme 3



Formula 26 Formula 27

Formula 10

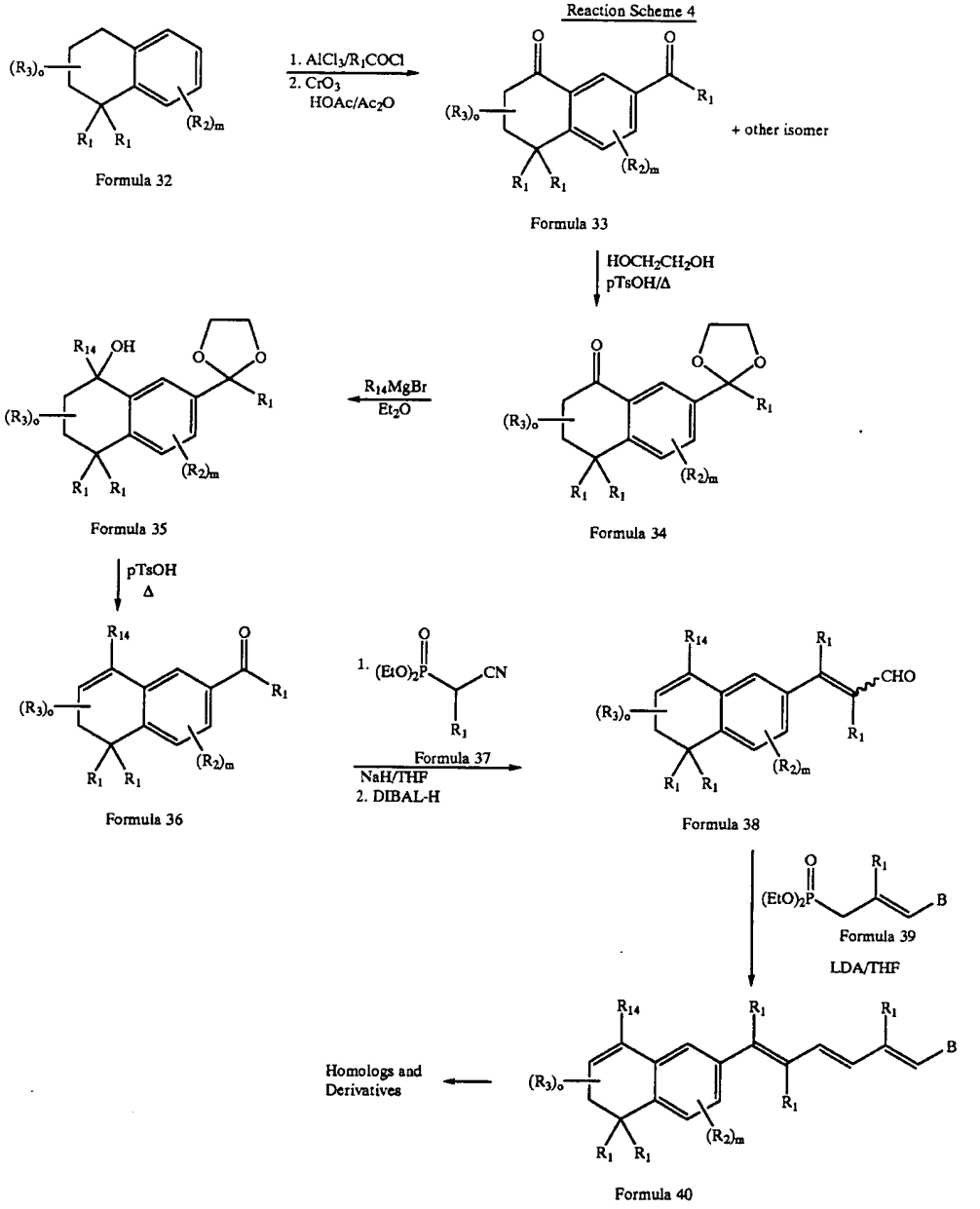
Formula 29 Formula 28

Formula 30 Formula 31

Homologs and Derivatives

Reaction Scheme 3 discloses a synthetic route to compounds where, with reference to Formula 1, X is $[C(R_1)_2]_n$, n is 0 and the Z group is an ethynyl function (—C≡C—). In accordance with this scheme, a 6-bromo-2,3-dihydro-1H-inden-1-one derivative of Formula 26 is reacted in a sequence of reactions (starting with reaction with trimethylsilylacetylene) which are analogous to the reactions described above in connection with Reaction Schemes 1 and 2, to provide, through intermediates of the formulas 27–30, the indene derivatives of Formula 31. In a preferred embodiment within the scope of Reaction Scheme 3, the starting material is 6-bromo-2,3-dihydro-3,3-dimethyl-1H-inden-1-one that is available in accordance with the chemical literature (See Smith et al. Org. Prep. Proced. Int. 1978 10, 123–131). Compounds of Formula 26, such as 6-bromo-2,3-dihydro-3,3-dimethyl-1H-inden-1-one, can also be used for the synthesis of still further exemplary compounds for use in the present invention, as described below.

Reaction Scheme 4



Formula 32

Formula 33

Formula 34

Formula 35

Formula 36

Formula 38

Formula 39

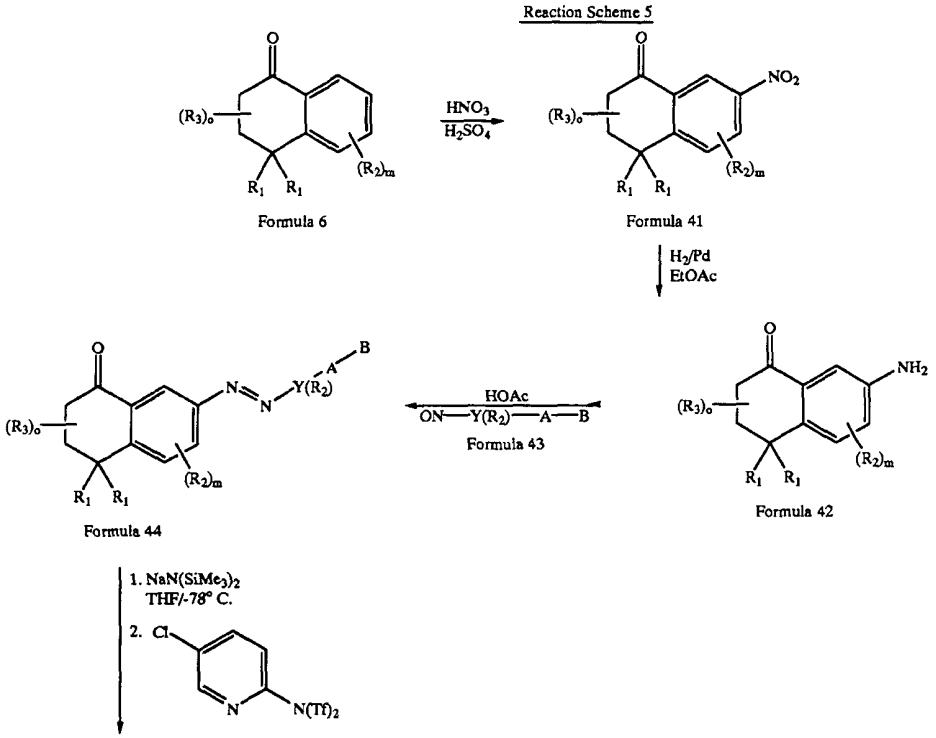
Formula 40

Referring now to Reaction Scheme 4 a synthetic route to exemplary compounds is disclosed where, with reference to Formula 1, Z is $—(CR_1{=}CR_1)_{n'}—$, n' is 3, 4 or 5 and Y represents a direct valence bond between the $(CR_1{=}CR_1)_{n'}$ group and B. This synthetic route is described for examples where the X group is $[C(R_1)_2]_n$ and n is 1

(dihydronaphthalene derivatives). Nevertheless, it should be understood that the reactions and synthetic methodology described in Reaction Scheme 4 and further ensuing schemes, is also applicable, with such modifications which will be readily apparent to those skilled in the art, to derivatives where X is is S, O, NR' (benzothiopyran, benzopyran or dihydroquinoline derivatives) or $[C(R_1)_2]_n$ and n is 0 (indene derivatives).

In accordance with Reaction Scheme 4, a 1,2,3,4-tetrahydronaphthalene derivative of Formula 32 is reacted with an acid chloride (R_1COCl) under Friedel Crafts conditions, and the resulting acetylated product is oxidized, for example in a Jones oxidation reaction, to yield a mixture of isomeric 6- and 7-acetyl-1(2H)-naphthalenone derivatives of Formula 33. In a specific preferred example of this reaction, the starting compound of Formula 32 is 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (a known compound) which can be prepared in accordance with a process described in the experimental section of the present application. The 7-acetyl-1(2H)-naphthalenone derivative of Formula 33 is reacted with ethylene glycol in the presence of acid to protect the oxo function of the exocyclic ketone moiety, as a ketal derivative of Formula 34. The ketal of Formula 34 is thereafter reacted with a Grignard reagent of the formula $R_{14}MgBr$ (the symbols are defined as in connection with Formula 1), to yield the tertiary alcohol of Formula 35. Thereafter the dioxolane protective group is removed and the tertiary alcohol is dehydrated by treatment with acid to provide the 3,4-dihydro-7-acetylnaphthalene derivatives of Formula 36. The ketone function of the compounds of Formula 36 is subjeceted to a Horner Emmons (or analogous) reaction under strongly alkaline conditions with a phosphonate reagent of Formula 37, to yield, after reduction, the aldehyde compounds of Formula 38. Still another Horner Emmons (or analogous) reaction under strongly alkaline conditions with a reagent of Formula 39 provides compounds of Formula 40. The latter can be converted into further homologs and derivatives in accordance with the reactions described above. A specific example of the Horner Emmons reagent of Formula 37 which is used for the preparation of a preferred compound is diethylcyanomethylphosphonate; an example of the Horner Emmons reagent of Formula 39 is diethyl-(E)-3-ethoxycarbonyl-2-methylallylphosphonate.

Reaction Scheme 5



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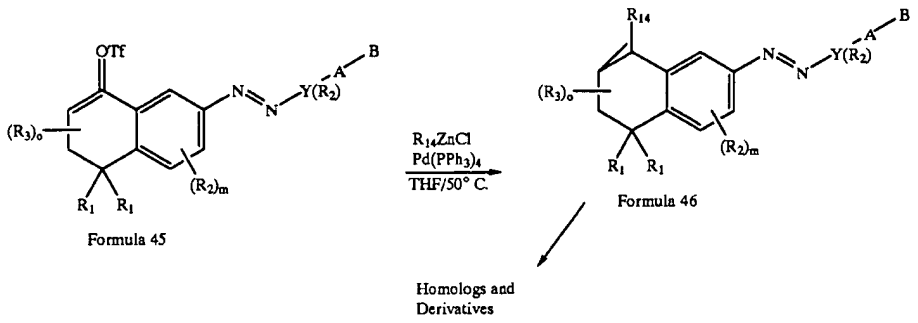
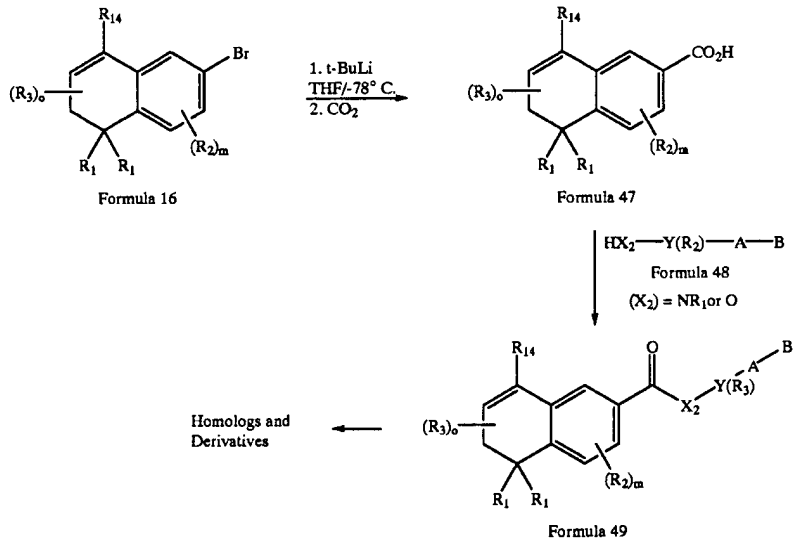


Formula 45

Formula 46

Reaction Scheme 5 discloses a synthetic process for preparing compounds where the Z group is an azo group (—N=N—). As in Reaction Scheme 4 this process is described for examples where the X group is $[C(R_1)_2]_n$ and n is 1 (dihydronaphthalene derivatives). Nevertheless, it should be understood that the synthetic methodology described is also applicable, with such modifications which will be readily apparent to those skilled in the art, to all azo compounds for use in the invention, namely to derivatives where X is is S, O, NR' (benzothiopyran, benzopyran or dihydroquinoline derivatives) or $[C(R_1)_2]_n$ and n is 0 (indene derivatives). Thus, a nitro group is introduced into the starting compound of Formula 6 under substantially standard conditions of nitration, to yield the 3,4-dihydro-7-nitro-1(2H)-naphthalenone derivative of Formula 41. The latter compound is reduced to the 3,4-dihydro-7-amino-1(2H)-naphthalenone derivative of Formula 42 and is thereafter reacted with a nitroso compound of the formula ON-$Y(R_2)$-A-B (Formula 43) under conditions normally employed (glacial acetic acid) for preparing azo compounds. The nitroso compound of Formula 43 can be obtained in accordance with reactions known in the art. A specific example for such compound, which is used for the synthesis of a preferred compound is ethyl 4-nitrosobenzoate. The azo compound of Formula 44 is thereafter reacted with sodium bis(trimethylsilyl)amide and 2-[N,N-bis (trifluoromethylsulfonyl)amino]-5-chloropyridine to yield the 4-trifluoromethylsulfonyloxy derivatives represented in Formula 45. The compounds of Formula 45 are then converted to the azo compounds shown in Formula 46, by reaction with an organometalic derivative derived from the aryl or heteroaryl compound $R_{14}H$. These latter two reactions, namely the conversion to the 4-trifluoromethylsulfonyloxy derivatives and subsequent reaction with the organometal derivative, have been described above in connection with Reaction Schemes 1, 2 and 3, and are employed in several presently preferred synthetic processes leading to exemplary RAR antagonist compounds.

Reaction Scheme 6



Formula 16

Formula 47

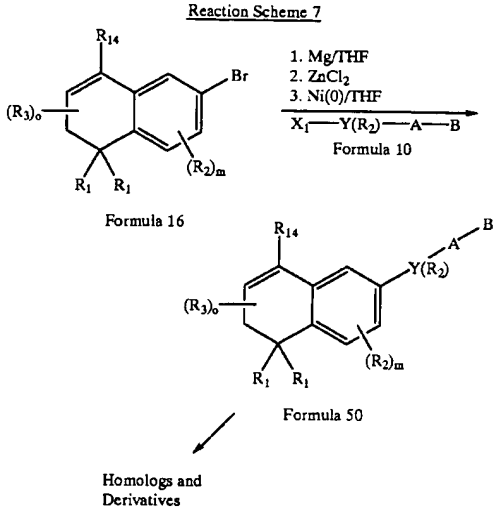
Formula 49

Reaction Scheme 6 discloses a presently preferred synthetic process for the preparation of compounds where, with reference to Formula 1, the Z group is COO— or $CONR_1$ (R_1 is preferably H). These ester and amide derivatives are prepared from the 3,4-dihydro-7-bromonaphthalene derivatives of Formula 16, which can be obtained as described in Reaction Scheme 1. Thus, the compounds of Formula 16 are

reacted with strong base, such as t-butyllithium, in an inert ether type solvent, such as tetrahydrofuran, at cold temperature, and carbon dioxide (CO_2) is added to provide the 5,6-dihydro-2-naphthalenecarboxylic acid derivatives of Formula 47. Compounds of Formula 47 are then reacted with compounds of the formula X_2-$Y(R_2)$-A-B (Formula 48) where X_2 reperesent an OH or an NR_1 group, the R_1 preferably being hydrogen. Those skilled in the art will recognize that the compounds of Formula 48 are aryl or heteroaryl hydroxy or amino derivatives which can be obtained in accordance with the state-of-the-art. The reaction between the compounds of Formula 47 and Formula 48 can be conducted under various known ester or amide forming conditions, such as coupling of the two in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 4-dimethylaminopyridine. Alternatively, the compounds of Formula 47 can be converted into the corresponding acid chloride for coupling with the compounds of Formula 48 in the presence of base. The amide or ester compounds of Formula 49 can be converted into further homologs and derivatives, as described above. Although Reaction Scheme 6 is described and shown for the example where the X group of Formula 1 is $[C(R_1)_2]_n$ and n is 1 (dihydronaphthalene derivatives), the herein described process can be adapted for the preparation of benzopyran, benzothiopyran, dihydroquinoline and indene derivatives as well.

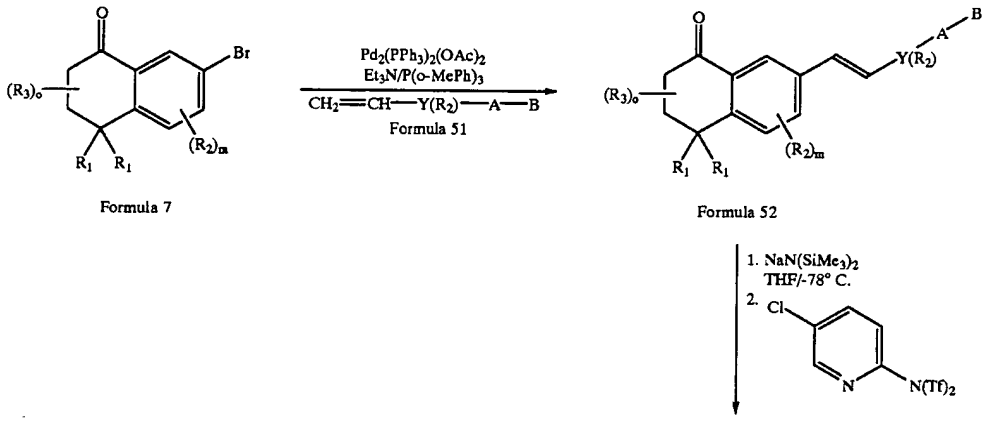
Compounds of the present invention where with reference to Formula 1, Z is —OCO—, NR_1CO, as well as the corresponding thioester and thioamide analogs, can be prepared from the intermediates derived from the compounds of Formula 16 where the bromo function is replaced with an amino or hydroxyl group and in accordance with the teachings of U.S. Pat. Nos. 5,324,744, the specification of which is expressly incorporated herein by reference.

Reaction Scheme 7



Reaction Scheme 7 discloses a presently preferred synthetic process for the preparation of compounds where with reference to Formula 1, Z is $—(CR_1{=}CR_1)_{n'}—$ and n' is 0. These compounds of Formula 50 can be obtained in a coupling reaction between compounds of Formula 16 and a Grignard reagent derived from the halo compounds of Formula 10. The coupling reaction is typically conducted in the presence of a zinc salt and a nickel (Ni(0)) catalyst in inert ether type solvent, such as tetrahydrofuran. The compounds of Formula 50 can be converted into further homologs and derivatives, as described above.

Reaction Scheme 8



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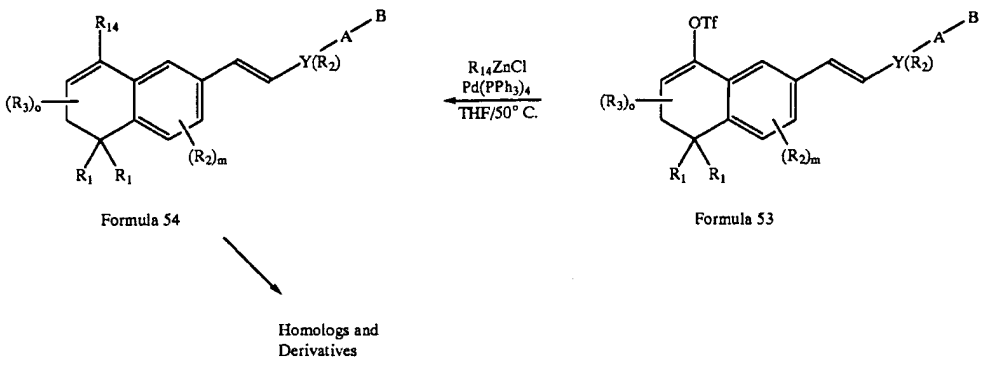


Referring now to Reaction Scheme 8 a presently preferred synthetic process is disclosed for the preparation of compounds where Z is $—(CR_1=CR_1)_{n'}—$ and n' is 1. More particularly, Reaction Scheme 8 discloses the presently preferred process for preparing those compounds which are dihydronaphtalene derivatives and where the Z group represents a vinyl (—CH=CH—) function. However, the generic methodology disclosed herein can be extended, with such modifications which will be apparent to those skilled in the art to the analogous benzopyran, benzothiopyran, dihydroquinoline compounds, and to compounds where the vinyl group is substituted. Thus, in accordance with Reaction Scheme 8 the 7-bromo-1(2H)-naphthalenone derivative of Formula 7 is reacted with a vinyl derivative of the structure $—CH_2=CH—Y(R_2)$-A-B (Formula 51) in the presence of a suitable catalyst, typically having the formula $Pd(PPh_3)$, an acid acceptor (such as triethylamine) under an inert gas (argon) atmosphere. The conditions of this reaction are analogous to the coupling of the acetylene derivatives of Formula 9 with the reagent of Formula 10 (see for example Reaction Scheme 1), and this type of reaction is generally known in the art as a Heck reaction. The vinyl derivative of Formula 51 can be obtained in accordance with the state of the art, an example for such a reagent used for the synthesis of a preferred compound to be used in the invention is ethyl 4-vinylbenzoate.

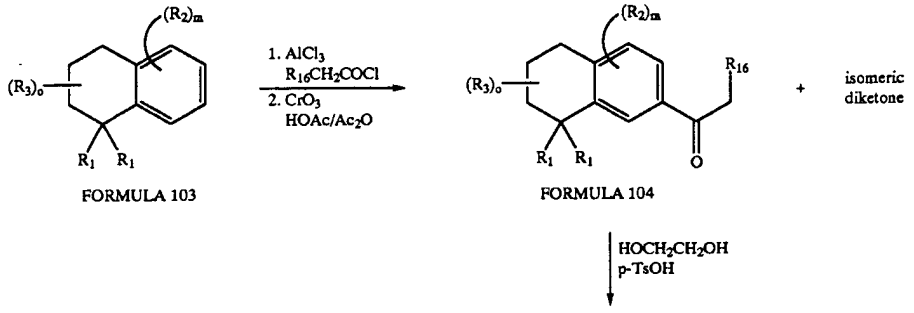
The product of the Heck coupling reaction is an ethenyl derivative of Formula 52, which is thereafter converted into compounds used in the present invention by treatment with sodium bis(trimethylsilyl)amide and 2-[N,N-bis (trifluoromethylsulfonyl)amino]-5-chloropyridine to yield the 4-trifluoromethylsulfonyloxy derivatives of Formula 53, and subsequent reaction with an organometal derivative derived from the aryl or heteroaryl compound $R_{14}H$, as described above. The resulting compounds of Formula 54 can be converted into further homologs and derivatives.

The compounds of Formula 54 can also be obtained through synthetic schemes which employ a Wittig or Horner Emmons reaction. For example, the intermediate of Formula 33 (see Reaction Scheme 4) can be reacted with a triphenylphosphonium bromide (Wittig) reagent or more preferably with a diethylphosphonate (Horner Emmons) reagent of the structure $(EtO)_2PO—CH_2—Y(R_2)$-A-B, as described for analogous Horner Emmons reactions in U.S. Pat. No. 5,324,840, the specification of which is incorporated herein by reference. The just mentioned Horner Emmons reaction provides intermediate compounds analogous in structure to Formula 52, and can be converted into compounds of Formula 54 by the sequence of reactions described in Reaction Scheme 8 for the compounds of Formula 52.

Synthetic Methods—Aryl and (3-Oxy-1-Propenyl)-Substituted Compounds

The exemplary RAR antagonist compounds of Formula 101 can be made by the synthetic chemical pathways illustrated here. The synthetic chemist will readily appreciate that the conditions set out here are specific embodiments which can be generalized to any and all of the compounds represented by Formula 101.

Reaction Scheme 101



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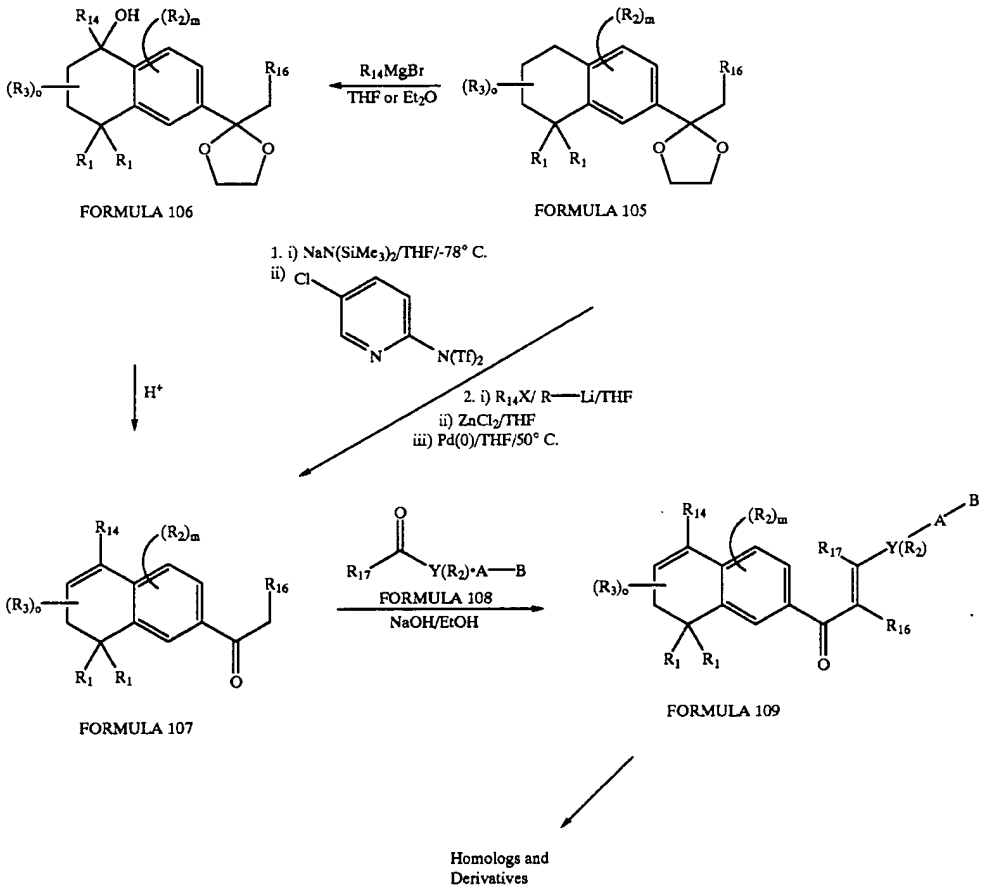


Reaction Scheme 101 illustrates the synthesis of compounds of Formula 101 where X is $[C(R_1)_2]_n$, n is 1, p is zero and R_{17} is H or lower alkyl. In other words, Reaction Scheme 101 illustrates the synthesis of compounds of the invention which are 3,4-dihydronaphthalene derivatives. In accordance with this scheme, a tetrahydronaphthalene compound of Formula 103 which is appropriately substituted with the R_3 and R_2 groups (as these are defined in connection with Formula 101) serves as the starting material. A preferred example of a compound of Formula 103 is 1,3,3,4-tetrahydro-1,1-dimethyl-naphthalene, which is described in the chemical literature (Mathur et al. *Tetrahedron*, 1985, 41:1509. A presently preferred route for the synthesis of this compound from 1-bromo-3-phenylpropane is also described in the experimental section of the present application.

The compound of Formula 103 is reacted in a Friedel Crafts type reaction with an acid chloride having the structure $R_{16}CH_2COCl$ (R_{16} is defined as in connection with Formula 101) and is thereafter oxidized with chromium trioxide in acetic acid to provide the isomeric 6 and 7 acyl-3,4-dihydro-1(2H)-naphthalenone derivatives. Only the 6-acyl derivative which is of interest from the standpoint of the present invention, is shown by structural formula (Formula 104) in Reaction Scheme 101. In the preparation of the presently preferred compounds of this invention the R_1 groups represent methyl, R_2, R_3 and R_{16} are H, and therefore the preferred intermediate corresponding to Formula 104 is 3,4-dihydro-4,4-dimethyl-6-acetyl-1(2H)-naphthalenone.

The exocyclic ketone function of the compound of Formula 104 is thereafter protected as a ketal, for example by treatment with ethylene glycol in acid, to provide the 1,3-dioxolanyl derivative of Formula 105. The compound of Formula 105 is then reacted with a Grignard reagent of the formula $R_{14}MgBr$ (R_{14} is defined as in connection with Formula 101) to give the 1,2,3,4-tetrahydro-1-hydroxy-naphthalene derivative of Formula 106. The exocyclic ketone function of the compound of Formula 106 is then deprotected by treatment with acid and dehydrated to give the compound of Formula 107.

An alternate method for obtaining the compounds of Formula 107 from the compounds of Formula 105 is by reacting the compounds of Formula 105 with sodium bis (trimethylsilyl)amide and 2-[N,N-bis (trifluoromethylsulfonyl)amino]-5-chloropyridine (Tf=SO_2CF_3) in an inert ether type solvent, such as tetrahydrofuran, at low temperatures (-78° C. and 0° C.). This reaction proceeds through a sodium salt intermediate which is usually not isolated and is not shown in Reaction Scheme 101. The overall reaction results in a trifluoromethylsulfonyloxy derivative, which is thereafter reacted with an organometal derivative derived from the aryl or heteroaryl compound $R_{14}H$, such that the formula of the organometal derivative is $R_{14}Met$ (Met stands for monovalent metal),

preferably $R_{14}Li$. (R_{14} is defined as in connection with Formula 101.) The reaction with the organometal derivative, preferably lithium derivative of the formula $R_{14}Li$ is usually conducted in an inert ether type solvent (such as tetrahydrofuran) in the presence of zinc chloride ($ZnCl_2$) and tetrakis(triphenylphosphine)-palladium(0) ($Pd(PPh_3)_4$). The organolithium reagent $R_{14}Li$, if not commercially available, can be prepared from the compound $R_{14}H$ (or its halogen derivative $R_{14}—X_1$ where X_1 is halogen) in an ether type solvent in accordance with known practice in the art. The temperature range for the reaction between the reagent $R_{14}Li$ and the trifluoromethylsulfonyloxy derivative is, generally speaking, in the range of approximately -78° C. to 50° C.

The compounds of the invention are formed as a result of a condensation between the ketone compound of Formula 107 and an aldehyde or ketone of Formula 108. In the preparation of the preferred exemplary compounds of the invention the reagent of Formula 108 is 4-carboxybenzaldehyde ($R_{17}—H$). Examples of other reagents within the scope of Formula 108 and suitable for the condensation reaction and for the synthesis of compounds within the scope of the present invention (Formula 101) are: 5-carboxy-pyridine-2-aldehyde, 4-carboxy-pyridine-2-aldehyde, 4-carboxy-thiophene-2-aldehyde, 5-carboxy-thiophene-2-aldehyde, 4-carboxy-furan-2-aldehyde, 5-carboxy-furan-2-aldehyde, 4-carboxyacetophenone, 2-acetyl-pyridine-5-carboxylic acid, 2-acetyl-pyridine-4-carboxylic acid, 2-acetyl-thiophene-4-carboxylic acid, 2-acetyl-thiophene-5-carboxylic acid, 2-acetyl-furan-4-carboxylic acid, and 2-acetyl-furan-5-carboxylic acid. The latter compounds are available in accordance with the chemical literature; see for example Decroix et al., *J Chem. Res.*(S), 4: 134 (1978); Dawson et al., *J Med. Chem.* 29:1282 (1983); and Queguiner et al., *Bull Soc. Chimique de France* No. 10, pp. 3678–3683 (1969). The condensation reaction between the compounds of Formula 107 and Formula 108 is conducted in the presence of base in an alcoholic solvent. Preferably, the reaction is conducted in ethanol in the presence of sodium hydroxide. Those skilled in the art will recognize this condensation reaction as an aldol condensation, and in case of the herein described preferred examples (condensing a ketone of Formula 107 with an aldehyde of Formula 108) as a Claisen-Schmidt reaction. (See March: *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure,* pp. 694–695 McGraw Hill (1968). The compounds of Formula 109 are within the scope of the present invention, and can also be subjected to further transformations resulting in additional compounds of the invention. Alternatively, the A-B group of Formula 108 may be a group which is within the scope of the invention, as defined in Formula 101, only after one or more synthetic transformations of such a nature which is well known and within the skill of the practicing organic chemist. For example, the reaction performed on the A-B group may be a deprotection step, homologation, esterification, saponification, amide formation or the like.

Generally speaking, regarding derivatization of compounds of Formula 109 and/or the synthesis of aryl and heteroaryl compounds of Formula 108 which can thereafter be reacted with compounds of Formula 107, the following well known and published general principles and synthetic methodology can be employed.

As indicated above, carboxylic acids are typically esterified by refluxing the acid in a solution of the appropriate alcohol in the presence of an acid catalyst such as hydrogen chloride or thionyl chloride. Alternatively, the carboxylic acid can be condensed with the appropriate alcohol in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine. The ester is recovered and purified by conventional means. Acetals and ketals are readily made by the method described in March, *Advanced Organic Chemistry* 2nd Edition, McGraw-Hill Book Company, p. 810). Alcohols, aldehydes and ketones all may be protected by forming respectively, ethers and esters, acetals or ketals by known methods such as those described in McOmie, Plenum Publishing Press, 1973 and Protecting Groups, Ed. Greene, John Wiley & Sons, 1981.

To increase the value of n in the compounds of Formula 108 before affecting the condensation reaction of Reaction Scheme 101 (where such compounds corresponding to Formula 108 are not available from a commercial source) aromatic or heteroaromatic carboxylic acids may be subjected to homologation (while the aldehyde group is protected) by successive treatment under Arndt-Eistert conditions or other homologation procedures. Alternatively, derivatives which are not carboxylic acids may also be homologated by appropriate procedures. The homologated acids can then be esterified by the general procedure outlined in the preceding paragraph.

Compounds of Formula 108, (or other intermediates or of the invention, as applicable) where A is an alkenyl group having one or more double bonds can be made for example, by synthetic schemes well known to the practicing organic chemist; for example by Wittig and like reactions, or by introduction of a double bond by elimination of halogen from an alpha-halo-arylalkyl-carboxylic acid, ester or like carboxaldehyde. Compounds of Formula 108 (or other intermediates or of the invention, as applicable) where the A group has a triple (acetylenic) bond can be made by reaction of a corresponding aromatic methyl ketone with strong base, such as lithium diisopropylamide, reaction with diethyl chlorophosphate and subsequent addition of lithium diisopropylamide.

The acids and salts derived from compounds of Formula 109 (or other intermediates or compounds of the invention, as applicable) are readily obtainable directly as a result of the condensation reaction, or from the corresponding esters. Basic saponification with an alkali metal base will provide the acid. For example, an ester of Formula 109 (or other intermediates or compounds of the invention, as applicable) may be dissolved in a polar solvent such as an alkanol, preferably under an inert atmosphere at room temperature, with about a three molar excess of base, for example, lithium hydroxide or potassium hydroxide. The solution is stirred for an extended period of time, between 15 and 20 hours, cooled, acidified and the hydrolysate recovered by conventional means.

The amide may be formed by any appropriate amidation means known in the art from the corresponding esters or carboxylic acids. One way to prepare such compounds is to convert an acid to an acid chloride and then treat that compound with ammonium hydroxide or an appropriate amine.

Alcohols are made by converting the corresponding acids to the acid chloride with thionyl chloride or other means (J. March, *Advanced Organic Chemistry,* 2nd Edition, McGraw-Hill Book Company), then reducing the acid chloride with sodium borohydride (March, Ibid, p. 1124), which gives the corresponding alcohols. Alternatively, esters may be reduced with lithium aluminum hydride at reduced temperatures. Alkylating these alcohols with appropriate alky halides under Williamson reaction conditions (March, Ibid,

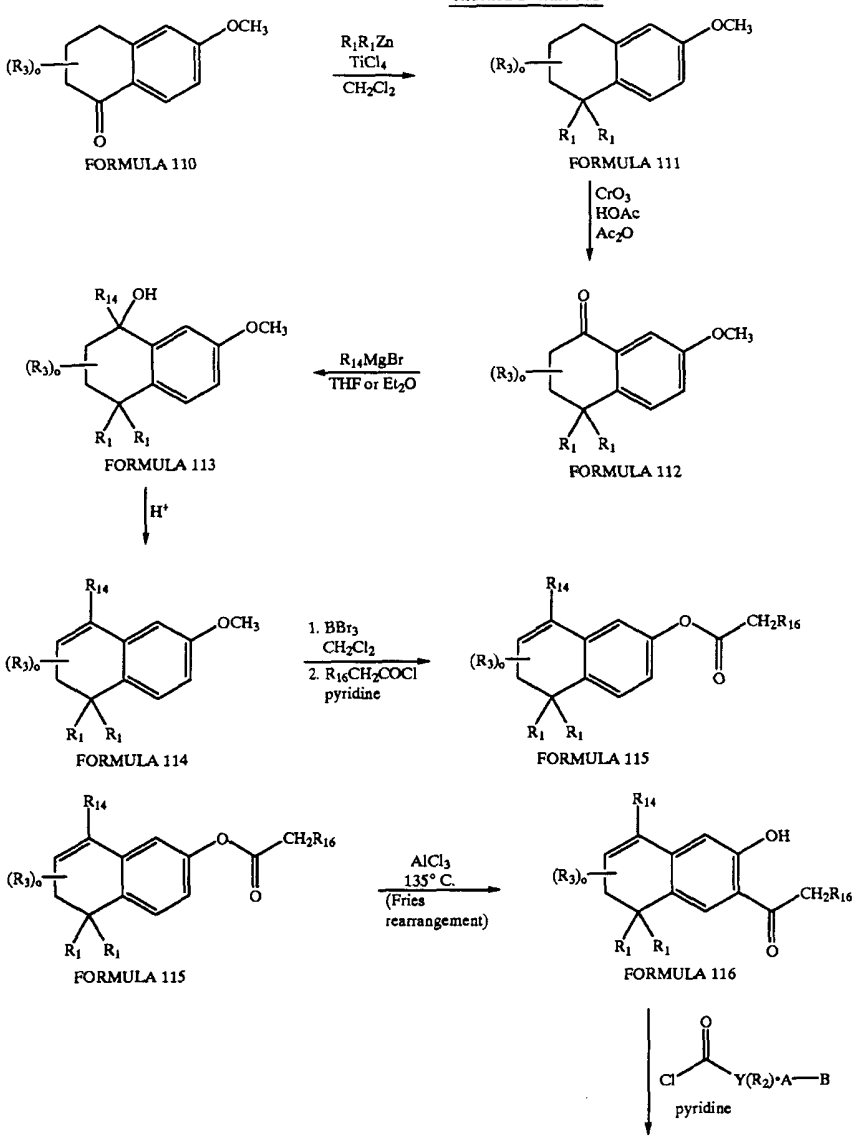
p. 357) gives the corresponding ethers. These alcohols can be converted to esters by reacting them with appropriate acids in the presence of acid catalysts or dicyclohexylcarbodiimide and dimethylaminopyridine.

Aldehydes can be prepared from the corresponding primary alcohols using mild oxidizing agents such as pyridinium dichromate in methylene chloride (Corey, E. J., Schmidt, G., *Tet. Lett.*, 399, 1979), or dimethyl sulfoxide/oxalyl chloride in methylene chloride (Omura, K., Swern, D., *Tetrahedron*, 34:1651 (1978)).

Ketones can be prepared from an appropriate aldehyde by treating the aldehyde with an alkyl Grignard reagent or similar reagent followed by oxidation.

Acetals or ketals can be prepared from the corresponding aldehyde or ketone by the method described in March, Ibid, p. 810.

Reaction Scheme 102



FORMULA 110

FORMULA 111

FORMULA 113

FORMULA 112

FORMULA 114

FORMULA 115

FORMULA 115

FORMULA 116

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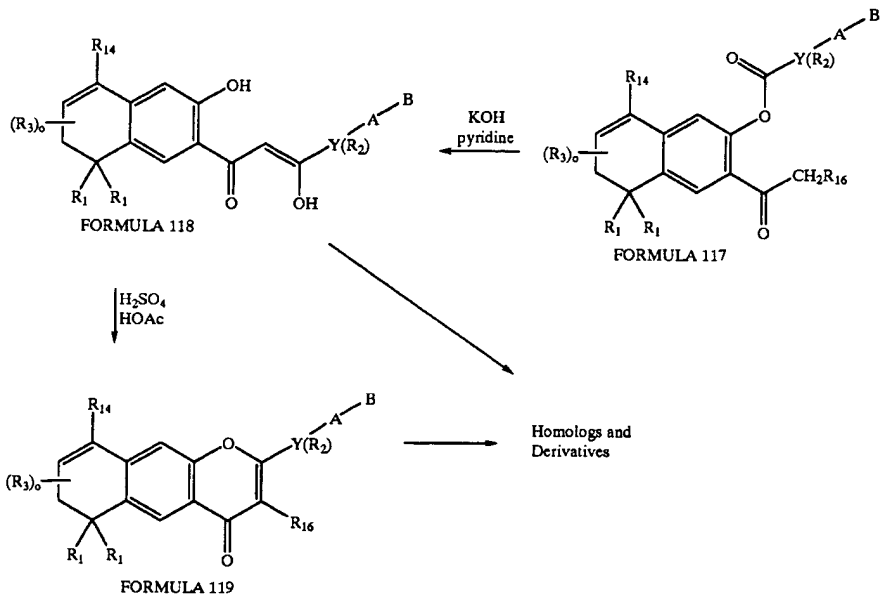


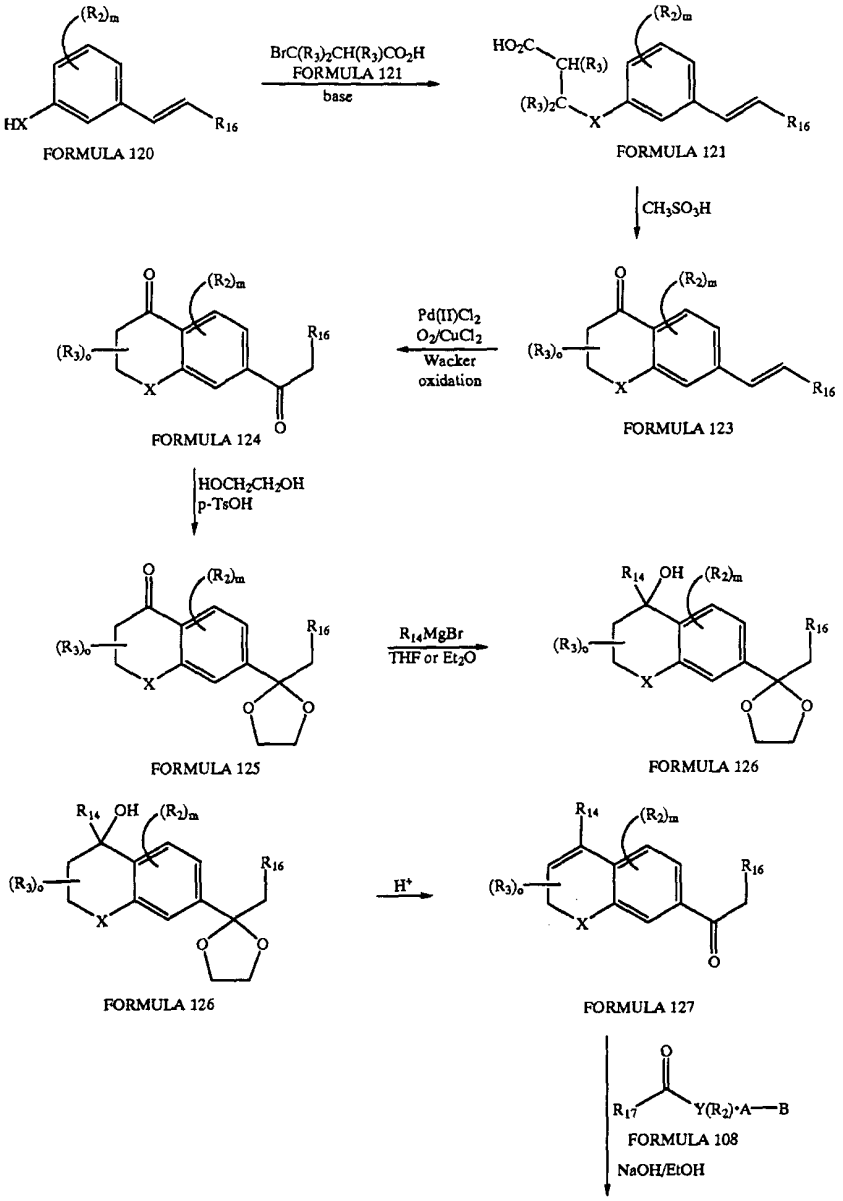
Referring now to Reaction Scheme 102, a synthetic route to those compounds of the invention is described in which, with reference to Formula 101 p is zero, R_2 in the aromatic portion of the condensed ring structure is OH and R_{17} is OH. Those skilled in the art will readily recognize that these compounds are β-diketones in the enol form. Reaction Scheme 102 also describes a synthetic route to those compounds of the invention where p is 1. Those skilled in the art will readily recognize that the latter compounds are flavones. Thus, in accordance with this scheme a 1,2,3,4-tetrahydro-6-methoxynaphthalene-1-one derivative of Formula 110 is reacted with dialkyl zinc (R_1Zn) in the presence of titanium tetrachloride in a suitable solvent such as CH_2Cl_2 to replace the oxo function with the geminal dialkyl group R_1R_1, to yield a compound of Formula 111, where R_1 is lower alkyl. In preferred embodiments of the compounds of the invention which are made in accordance with Reaction Scheme 102 the R_3 group is hydrogen and R_1 are methyl. Accordingly, the dialkyl zinc reagent is dimethyl zinc, and the preferred starting material of Formula 110 is 1,2,3,4-tetrahydro-6-methoxynaphthalene-1-one. The latter compound is commercially available, for example from Aldrich Chemical Company. The 1,2,3,4-tetrahydro-1,2-dialkyl-6-methoxy naphthalene derivative of Formula 111 is thereafter oxidized with chromium trioxide in acetic acid and acetic anhydride to give a 1,2,3,4-tetrahydro-3,4-dialkyl-7-methoxy naphthalen-1-one derivative of Formula 112. The ketone compound of Formula 112 is reacted with a Grignard reagent ($R_{14}MgBr$, R_{14} is defined as in connection with Formula 101) to yield a 1-hydroxy-1-aryl-3,4-dihydro-3,4-dialkyl-7-methoxy naphthalene derivative of Formula 113. The hydroxy compound of Formula 113 is subjected to elimination by heating, preferably in acid, to yield the dihydronaphthalene compound of Formula 114. The methyl group is removed from the phenolic methyl ether function of the compound of Formula 114 by treatment with boron tribromide in a suitable solvent, such as CH_2Cl_2, and therafter the phenolic OH is acylated with an acylating agent that introduces the $R_{16}CH_2CO$ group, to give a compound of Formula 115. In the preferred embodiment R_{16} is H, and therefore the acylating agent is acetyl chloride or acetic anhydride. The acetylation reaction is conducted in a basic solvent, such as pyridine. The acylated phenol compound of Formula 115 is reacted with aluminum chloride at elevated temperature, causing it to undergo a Fries rearrangement and yield the 1-aryl-3,4-dialkyl-3,4-dihydro-6-acyl-7-hydroxy-naphthalene compound of Formula 116. The phenolic hydroxyl group of the compound of Formula 116 is acylated with an acylating agent (such as an acid chloride) that introduces the CO—$Y(R_2)$-A-B group to yield a compound of Formula 117. In the acid chloride reagent Cl—CO—$Y(R_2)$-A-B (or like acylating reagent) the symbols Y, R_2 and A-B have the meaning defined in connection with Formula 101. In the preparation of a preferred compound of the invention in accordance with this scheme this reagent is $ClCOC_6H_4COOEt$ (the half ethyl ester half acid chloride of terephthalic acid).

The compound of Formula 117 is reacted with strong base, such as potassium hydroxyde in pyridine, to yield, as a result of an intramolecular Claisen condensation reaction, a compound of Formula 118. The compounds of Formula 118 are within the scope of the invention and of Formula 101, where there is an OH for the R_2 substituent in the aromatic portion of the condensed ring moiety and R_{17} is OH. In connection with the foregoing reaction (intramolecular Claisen condensation) and the previously mentioned Fries rearrangement it is noted that these probable reaction mechanisms are mentioned in this description for the purpose of fully explaining the herein described reactions, and for facilitating the work of a person of ordinary skill in the art to perform the herein described reactions and prepare the compounds of the invention. Nevertheless, the present inventors do not wish to be bound by reaction mechanisms and theories, and the herein claimed invention should not be limited thereby.

The compounds of Formula 118 are reacted with strong acid, such as sulfuric acid, in a suitable protonic solvent, such as acetic acid, to yield the flavone compounds of

Formula 119. The compounds of Formula 119 are also compounds of the invention, within the scope of Formula 101 where p is 1. Both the compounds of Formula 118 and Formula 119 can be subjected to further reactions and transformations to provide further homologs and derivatives, as described above in connection with Reaction Scheme 101. This is indicated in Reaction Scheme 102 as conversion to homologs and derivatives.

Reaction Scheme 103



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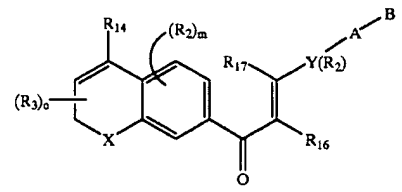
Homologs and Derivatives



FORMULA 128

Referring now to Reaction Scheme 103 a synthetic route is shown leading to those compounds of the invention where, with reference to Formula 101 X is S, O or NR', p is zero and R_{17} is H or lower alkyl. However, by applying the generic principles of synthesis shown in Reaction Scheme 102 the presently shown synthetic process can be modified or adapted by those of ordinary skill in the art to also obtain compounds of the invention where X is S, O or NR' and p is 1, or where X is S, O or NR' and p is zero, the R_2 group in the aromatic portion of the condensed ring moiety is OH and R_{17} is OH.

The starting compound of Reaction Scheme 103 is a phenol, thiophenol or aniline derivative of Formula 120. In the presently preferred compounds of the invention the R_2 and R_{16} groups are both hydrogen, and the preferred starting compounds of Formula 120 are 3-ethenyl-thiophenol or 3-ethenyl-phenol which are known in the chemical literature (Nuyken, et al. *Polym. Bull* (Berlin) 11:165 (1984). For the sake of simplifying the present specification, in the ensuing description X can be considered primarily sulfur as for the preparation of benzothiopyran derivatives of the present invention. It should be kept in mind, however, that the herein described scheme is also suitable, with such modifications which will be readily apparent to those skilled in the art, for the preparation of benzopyran (X=O) and dihydroquinoline (X=NR') compounds within the scope of the present invention. Thus, the compound of Formula 120 is reacted under basic condition with a 3-bromo carboxylic acid of the Formula 121. In this reaction scheme the symbols have the meaning described in connection with Formula 101. An example for the reagent of Formula 121 where R_3 is hydrogen, is 3-bromopropionic acid. The reaction with the 3-bromocarboxylic acid of Formula 121 results in the compound of Formula 122. The latter is cyclized by treatment with acid to yield the 7-ethenyl-thiochroman-4-one derivative (when X is S) or 7-ethenyl-chroman derivative (when X is 0) of Formula 123. The 7-ethenyl-thiochroman-4-one or 7-ethenyl-chroman-4-one derivative of Formula 123 is oxidized in the presence of $Pd(II)Cl_2$ and $CuCl_2$ catalysts to provide the corresponding 7-acyl (ketone) compound of Formula 124. Those skilled in the art will recognize the latter reaction as a Wacker oxidation. The exocyclic ketone group of the compound of Formula 124 is protected in the form of a ketal, for example by treatment with ethylene glycol in acid, to provide the 1,3-dioxolanyl derivative of Formula 125. Thereafter the compound of Formula 125 is subjected to a sequence of reactions analogous to those described for the compounds of Formula 105 in Reaction Scheme 101. Thus, the 1,3-dioxolanyl derivative of Formula 125 is reacted with a Grignard reagent of the formula $R_{14}MgBr$ to give the tertiary alcohol of Formula 126, which is thereafter dehydrated in acid to provide the benzothiopyran (X is S), benzopyran (X is O) or dihydroquinoline (X is NR') derivative of Formula 127. The ketone compound of Formula 127 is then reacted in the presence of base with the reagent of Formula 108 in an aldol condensation (Claisen-Schmidt) reaction to provide compounds of the invention of Formula 128. The compounds of Formula 128 can be converted into further homologs and derivatives, as described above in connection with Reaction Schemes 101 and 102.

Specific Examples

2-hydroxy-2-methyl-5-phenylpentane

To a mixture of magnesium turnings 13.16 g (0.541 mol) in 200 ml of anhydrous Et_2O was added 100.0 g (0.492 mol) of 1-bromo-3-phenyl propane as a solution in 100 ml of Et_2O. After of 5–10 ml of the solution had been added, the addition was stopped until the formation of the Grignard reagent was in progress. The remaining bromide was then added over 1 hour. The Grignard reagent was stirred for 20 minutes at 35° C. and then 31.64 g (0.541 mol) of acetone was added over a 45 minute period. The reaction was stirred overnight at room temperature before being cooled to 0° C. and acidified by the careful addition of 20% HCl. The aqueous layer was extracted with Et_2O (3×200 ml) and the combined organic layers washed with water, and saturated aqueous NaCl before being dried over $MgSO_4$. Removal of the solvent under reduced pressure and distillation of the residue afforded 63.0 g (72%) of the product as a pale-yellow oil, bp 99–102° C./0.5 mm Hg. 1H NMR ($CDCl_3$): δ 7.28–7.18 (5H, m), 2.63 (2H, t, J=7.5 Hz), 1.68 (2H, m), 1.52 (2H, m), 1.20 (6H, s).

1,2,3,4-tetrahydro-1,1-dimethylnaphthalene

A mixture of P_2O_5 (55.3 g, 0.390 mol) in 400 ml of methanesulfonic acid was heated to 105° C. under argon until all of the solid had dissolved. The resulting solution was cooled to room temperature and 2-hydroxy-2-methyl-5-phenylpentane (63.0 g, 0.354 mol) added slowly with stirring. After 4 hours the reaction was quenched by carefully pouring the solution onto 1 L of ice. The resulting mixture was extracted with Et_2O (4×125 ml) and the combined organic layers washed with water, saturated aqueous $NaHCO_3$, water, and saturated aqueous NaCl before being dried over $MgSO_4$. Concentration of the solution under reduced pressure, followed by distillation afforded 51.0 g (90%) of the product as a clear colorless oil, bp. 65–67° C./1.1 mmHg. 1H NMR ($CDCl_3$): δ 7.32 (1H, d, J=7.4 Hz), 7.16–7.05 (3H, m), 2.77 (2H, t, J=6.3 Hz), 1.80 (2H, m), 1.66 (2H, m), 1.28 (6H, s).

3,4-dihydro-4,4-dimethyl-1(2H)-naphthalenone (Compound A)

A solution of 350 ml of glacial acetic acid and 170 ml of acetic anhydride was cooled to 0° C. and CrO_3, 25.0 g (0.25 mol) carefully added in small portions. The resulting mixture was stirred for 30 minutes before 120 ml of benzene was added. 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene was added slowly as a solution in 30 ml of benzene. Upon completing the addition the reaction was stirred for 4 hours

at 0° C. The solution was diluted with H_2O (200 ml) and extracted with Et_2O (5×50 ml). The combined organic layers were washed with water, saturated aqueous $NaCO_3$, and saturated aqueous NaCl, before being dried over $MgSO_4$. Removal of the solvents under reduced pressure, and distillation afforded 16.0 g (74%) of the product as a pale-yellow oil, bp 93–96° C./0.3 mm Hg 1H NMR ($CDCl_3$): δ 8.02 (1H, dd, J=1.3, 7.8 Hz), 7.53 (1H, m), 7.42 (1H, d, J=7.9 Hz), 7.29 (1H, m), 2.74 (2H, t, J=6.8 Hz), 2.02 (2H, t, J=6.8 Hz), 1.40 (6H, s).

3,4-dihydro-4,4-dimethyl-7-bromo-1(2H)-naphthalenone (Compound B)

A 100 ml three-necked flask, fitted with an efficient reflux condenser and drying tube, and addition funnel, was charged with a mixture of $AlCl_3$ 9.5 g (71.4 mmol) and 3 ml of CH_2Cl_2. The 3,4-dihydro-4,4-dimethyl-1(2H)-naphthalenone (5.0 g, 28.7 mmol), was added dropwise with stirring (Caution: Exothermic Reaction!) to the mixture at room temperature. Bromine, 5.5 g (34.5 mmol), was then added very slowly, and the resulting mixture stirred for 2 hours at room temperature. (Note: if stirring stops, the mixture can be warmed to 70° C. until stirring resumes.) The reaction was then quenched by the slow addition of ice-cold 6M HCl. The mixture was extracted with Et_2O and the combined organic layers washed with water, saturated aqueous $NaHCO_3$, and saturated NaCl, before being dried over $MgSO_4$. Removal of the solvent under reduced pressure, and distillation of the residue afforded 5.8 g (80%) of the product as a pale-yellow oil which solidified on standing, bp: 140° C./0.4 mm Hg. 1H NMR ($CDCl_3$): δ 8.11 (1H, d, J=3.0 Hz), 7.61 (1H, dd, J=3.0, 9.0 Hz), 7.31 (1H, d, J=9.0 Hz), 2.72 (2H, t, J=6.0 Hz), 2.01 (2H, t, J=6.0 Hz), 1.28 (6H, s).

1,2,3,4-tetrahydro-1-hydroxy-1-(4-methylphenyl)-4,4-dimethyl-7-bromonaphthalene (Compound C)

To a mixture of magnesium turnings (648.0 mg, 27.0 mmol) in 25 ml of THF was added a solution of 4-bromotoluene (5.40 g, 31.8 mmol) in 10 ml of THF in two portions. The reaction was initiated by the addition of 2 ml of the solution, followed by the slow addition of the remaining solution via an addition funnel. The mixture was stirred at room temperature for 1 hour, and then the solution was transferred to a second flask using a canula. To the resulting Grignard reagent was added 4.0 g (15.9 mmol) of 3,4-dihydro-4,4-dimethyl-7-bromo-1(2H)-naphthalenone (Compound B) as a solution in 15 ml of THF. The resulting solution was heated to reflux overnight, cooled to room temperature, and the reaction quenched by the careful addition of ice-cold 10% HCl. Extraction with Et_2O was followed by washing of the combined organic layers with H_2O and saturated aqueous NaCl, then drying over $MgSO_4$. Removal of the solvent under reduced pressure provided an oil which afforded the product as a colorless solid after column chromatography (hexanes/EtOAc, 96:4). 1H NMR ($CDCl_3$): δ 7.36 (1H, dd, J=2.1, 7.6 Hz), 7.26 (3H, m), 7.12 (3H, s), 2.34 (3H, s), 2.24–2.04 (2H, m), 1.81 (1H, m), 1.55 (1H, m), 1.35 (3H, s), 1.30 (3H, s).

3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-7-bromonaphthalene (Compound D)

A flask equipped with a Dean-Stark trap was charged with 3.4 g of (9.85 mmol) of 1,2,3,4-tetrahydro-1-hydroxy-1-(4-methylphenyl)-4,4-dimethyl-7-bromonaphthalene (Compound C) and 40 ml of benzene. A catalytic amount of p-toluenesulfonic acid monohydrate was added and the resulting solution heated to reflux for 2 hours. Upon cooling to room temperature, Et_2O was added and the solution washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl then dried over $MgSO_4$. Removal of the solvents under reduced pressure, and column chromatography (100% hexane/silica gel) afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 7.32 (1H, dd, J=2.1, 8.2 Hz), 7.21 (5H, m), 7.15 (1H, d, J=2.1 Hz), 5.98 (1H, t, J=4.7 Hz), 2.40 (3H, s), 2.32 (2H, d, J=4.7 Hz), 1.30 (6H, s).

7-Ethynyl-3,4-dihydro-4,4-dimethylnaphthalen-1(2H)-one (Compound E)

To a solution (flushed for 15 minutes with a stream of argon) of 7 g (27.6 mmol) of 3,4-dihydro-4,4-dimethyl-7-bromo-1(2H)-naphthalenone (Compound B) in 150 ml of triethylamine was added 0.97 g (1.3 mmol) of bis (triphenylphosphine)palladium(II) chloride and 0.26 g (1.3 mmol) of cuprous iodide. The solution mixture was flushed with argon for 5 minutes and then 39 ml (36.6 mmol) of (trimethylsilyl)acetylene was added. The reaction mixture was sealed in a pressure tube and placed in a preheated oil bath (100° C.) for 24 hours. The reaction mixture was then filtered through Celite, washed with Et_2O and the filtrate concentrated in vacuo to give crude 7-(trimethylsilyl) ethynyl-3,4-dihydro-4,4-dimethylnaphthalen-1(2H)-one. To a solution of this crude TMS-acetylenic compound in 50 ml of methanol was added 0.6 g (4.3 mmol) of K_2CO_3. The mixture was stirred for 8 hours at ambient temperature and then filtered. The filtrate was concentrated in vacuo, diluted with Et_2O, washed with water, 10% HCl and brine, dried over $MgSO_4$ and concentrated in vacuo. Purification by column chromatography (silica, 10% EtOAc-hexane) yielded the title compound as a white solid. PMR ($CDCl_3$): δ 1.39 (6H, s), 2.02 (2H, t, J=7.0 Hz), 2.73 (2H, t, J=7.0 Hz), 3.08 (1H, s), 7.39 (1H, d, J=8.2 Hz), 7.61 (1H, dd, J=1.8 , 8.2 Hz), 8.14 (1H, d, J=9 1.8 Hz).

Ethyl-4-iodobenzoate

To a suspension of 10 g (40.32 mmol) of 4-iodobenzoic acid in 100 ml absolute ethanol was added 2 ml thionyl chloride and the mixture was then heated at reflux for 3 hours. Solvent was removed in vacuo and the residue was dissolved in 100 ml ether. The ether solution was washed with saturated $NaHCO_3$ and saturated NaCl solutions and dried ($MgSO_4$). Solvent was then removed in vacuo and the residue Kugelrohr distilled (100° C.; 0.55 mm) to give the title compound as a colorless oil, PMR ($CDCl_3$): δ 1.42 (3H, t, J~7 Hz), 4,4 (2H, q, J~7 Hz), 7.8 (4H).

6-iodonicotinic acid

Sodium iodide (20.59 g, 137.40 mmol) was cooled to -78° C. under argon and then hydriodic acid (97.13 g, 759.34 mmol) was added. The cooling bath was removed and the suspension was stirred for 5 minutes. To this mixture was added 6-chloronicotinic acid (22.09 g, 140.20 mmol) and the resulting mixture was slowly warmed to ambient temperature with stirring. The mixture was heated to reflux at 125° C. for 24 hours, cooled to ambient temperature and poured into acetone (500 ml) at 0° C. The yellow solid was collected by filtration and washed with 200 ml of 1N aqueous $NaHSO_3$ solution. Recrystallization from methanol (crystals were washed with ethyl ether) afforded the title compound as white crystals: mp 177–179° C. [lit. mp 187–192, Newkome et al. "Reductive Dehalogenation of Electron-Poor Heterocycles: Nicotinic Acid Derivatives" *J. Org. Chem.* 51: 953–954 (1986). 1H NMR (DMSO-d6): δ 8.81 (1H, dd, J=0.8, 2.4 Hz), 8.01 (1H, dd, J=0.8, 8.2 Hz), 7.91 (1H, dd, J=2.4, 8.2 Hz).

Ethyl 6-iodonicotinoate

To a suspension of 6-iodonicotinic acid (23.38 g, 94.20 mmol) in dichloromethane (100 ml) was added a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (19.86 g, 103.6 mmol) in dichloromethane (250 ml). To this mixture was added ethanol (12.40 g, 269.27

mmol) followed by dimethylaminopyridine (1.15 g, 9.41 mmol). The mixture was heated at 50° C. for 24.5 hours, concentrated in vacuo, and diluted with water (200 ml) then extracted with ethyl ether (550 ml). The combined organic phases were washed with saturated aqueous NaCl, dried ($MgSO_4$) and concentrated to a yellow solid. Purification by flash chromatography (silica, 10% EtOAc-hexane) afforded the title compound as white needles: mp 48–49° C.; 1H NMR ($CDCl_3$): δ 8.94 (1H, d, J=2.1 Hz), 7.91 (1H, dd, J=2.1, 8.2 Hz), 7.85 (1H, d, J=8.2 Hz), 4.41 (2H, q, J=7.1 Hz), 1.41 (3H, t, J=7.1 Hz).

Ethyl 4-[(5,6,7,8-tetrahydro-5,5-dimethyl-8-oxo-2-naphthalenyl)ethynyl]benzoate (Compound F)

To a solution of 4 g (21.7 mmol) of 7-ethynyl-3,4-dihydro-4,4-dimethylnaphthalen-1(2H)-one (Compound E) flushed for 15 minutes with a stream of argon, and 6 g (21.7 mmol) of ethyl 4-iodobenzoate in 100 ml of triethylamine was added 5 g (7.2 mmol) of bis(triphenylphosphine) palladium(II) chloride and 1.4 g (7.2 mmol) of cuprous iodide. The mixture was flushed with argon for 5 minutes and then stirred at ambient temperature for 18 hours. The reaction mixture was filtered through Celite and the filtrate was concentrated in vacuo. Purification by flash chromatography (silica, 10% EtOAc-hexane) yielded the title compound as a white solid. PMR ($CDCl_3$): δ 1.41 (3H, t, J=7.2 Hz), 1.41 (6H, s), 2.04 (2H, t, J=6.5 Hz), 2.76 (2H, t, J=6.5 Hz), 4.40 (2H, q, J=7.2 Hz), 7.44 (1H, d, J=8.2 Hz), 7.59 (2H, d, J=8.4 Hz), 7.68 (1H, dd, J=1.8, 8.2 Hz), 8.04 (2H, d, J=8.4 Hz), 8.15 (1H, d, J=1.8 Hz).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl] benzoate (Compound G)

To a cold solution (−78° C.) of 291.6 mg (1.59 mmol) of sodium bis(trimethylsily)amide in 5.6 ml of THF was added a solution of 500.0 mg (1.44 mmol) of ethyl 4-[(5,6,7,8-tetrahydro-5,5-dimethyl-8-oxo-2-naphthalenyl)ethynyl] benzoate (Compound F) in 4.0 ml of THF. The reaction mixture was stirred at −78° C. for 35 minutes and then a solution of 601.2 mg (1.59 mmol) of 5-chloro(2-bis-triflouromethylsulfonyl)imide in 4.0 ml of THF was added. After stirring at −78° C. for 1 hour, the solution was warmed to 0° C. and stirred for 2 hours. The reaction was quenched by the addition of saturated aqueous NH_4Cl. The mixture was extracted with EtOAc (50 ml) and the combined organic layers were washed with 5% aqueous NaOH, water, and brine. The organic phase was dried over Na_2SO_4 and then concentrated in vacuo to a yellow oil. Purification by column chromatography (silica, 7% EtOAc-hexanes) yielded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 8.04 (2H, dd, J=1.8, 8.4 Hz), 7.60 (2H, dd, J=1.8, 8.4 Hz), 7.51 (2H, m), 7.32 (1H, d, J=8.0 Hz), 4.40 (2H, q, J=7.1 Hz), 6.02 (1H, t, J=5.0 Hz), 2.44 (2H, d, J=5.0 Hz), 1.43 (3H, t, J=7.1 Hz), 1.33 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1)

A solution of 4-lithiotoluene was prepared by the addition of 189.9 mg (1.74 ml, 2.96 mmol) of t-butyl lithium (1.7M solution in hexanes) to a cold solution (−78° C.) of 253.6 mg (1.482 mmol) of 4-bromotoluene in 2.0 ml of THF. After stirring for 30 minutes a solution of 269.4 mg (1.977 mmol) of zinc chloride in 3.0 ml of THF was added. The resulting solution was warmed to room temperature, stirred for 30 minutes, and added via cannula to a solution of 472.9 mg (0.988 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl] benzoate (Compound G) and 50 mg (0.04 mmol) of tetrakis (triphenylphosphine)palladium(0) in 4.0 ml of THF. The resulting solution was heated at 50° C. for 45 minutes, cooled to room temperature and diluted with sat. aqueous NH_4Cl. The mixture was extracted with EtOAc (40 ml) and the combined organic layers were washed with water and brine. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to a yellow oil. Purification by column chromatography (silica, 5% EtOAc-hexanes) yielded the title compound as a colorless solid. 1H NMR (d6-acetone): δ 1.35 (6H, s), 1.40 (3H, t, J=7.1 Hz), 2.36 (2H, d, J=4.7 Hz), 2.42 (3H,s), 4.38 (2H, q, J=7.1 Hz), 5.99 (1H, t, J=4.7 Hz), 7.25 (5H, m), 7.35 (2H, m), 7.52 (2H, d, J=8.5 Hz), 7.98 (2H, d, J=8.5 Hz).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-phenyl-2-naphthalenyl)ethynyl]benzoate (Compound 1a)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 203.8 mg (0.43 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 58.2 mg (0.36 ml, 0.69 mmol) of phenyllithium (1.8M solution in cyclohexane/Et_2O), 116.1 mg (0.85 mmol) of zinc chloride and 13.8 mg (0.01 mmol) of tetrakis(triphenylphosphine) palladium(0). PMR ($CDCl_3$): δ 1.36 (6H, s), 1.40 (3H, t, J=7.1Hz), 2.37 (2H, d, J=4.7 Hz), 4.38 (2H, q, J=7.1 Hz), 6.02 (1H, t, J=4.7 Hz), 7.20 (1H, d, J=1.5 Hz), 7.27 (1H, m), 7.39 (6H, m), 7.52 (2H, d, J=8.2 Hz), 7.98 (2H, d, J=8.2 Hz).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 2)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 284.8 mg (2.090 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 3-methylphenyl lithium (prepared by adding 201.2 mg (1.86 ml, 3.14 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 274.0 mg (1.568 mmol) of 3-methylbromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.99 (2H, d, J=8.4 Hz), 7.51 (2H, d, J=8.4 Hz), 7.39–7.14 (7H, m), 5.99 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.60 (3H, s), 2.35 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 3)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 200.0 mg (0.418 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 199.4 mg (1.463 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 4.0 ml of THF, and 2-methylphenyl lithium (prepared by adding 133.9 mg (1.23 ml, 2.09 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 178.7 mg (1.045 mmol) of 2-methylbromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.97 (2H, d, J=8.4 Hz), 7.50 (2H, d, J=8.4 Hz), 7.49–7.19 (6H, m), 6.81 (1H, d, J=1.6 Hz), 5.89 (1H, t, J=4.5 Hz), 4.36 (2H, q, J=7.1 Hz), 2.43–2.14 (2H, dq, J=3.7, 5.4 Hz), 2.15 (3H, s), 1.39–1.34 (9H, m).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3,5-dimethylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 4)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 249.0 mg (1.827 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 3,5-dimethylphenyl lithium (prepared by adding 167.7 mg (1.54 ml, 2.62 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 249.0 mg (1.305 mmol) of 3,5-dimethylbromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.4 Hz), 7.52 (2H, d, J=8.4 Hz), 7.40–7.33 (2H, m), 7.20 (1H, d, J=1.6 Hz), 7.00 (1H, s), 6.97 (2H, s), 5.97 (1H, t, J=4.8 Hz), 4.37 (2H, q, J=7.1 Hz), 2.36 (6H, s), 2.34 (2H, d, J=4.8 Hz), 1.39 (3H, t, J=7.1 Hz), 1.37 (6H, s).

Ethyl4-[(5,6-dihydro-5,5-dimethyl-8-(4-ethylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 5)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 249.0 mg (1.827 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4-ethylphenyl lithium (prepared by adding 167.7 mg (1.54 ml, 2.62 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 244.0 mg (1.305 mmol) of 4-ethylbromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.99 (2H, d, J=8.4 Hz), 7.51 (2H, d, J=8.4 Hz), 7.42–7.24 (7H, m), 5.99 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.71 (2H, q, J =7.6 Hz), 2.35 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-(1,1-dimethylethyl)phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 6)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thiazolyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.52 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl] benzoate (Compound G) was converted into the title compound (colorless solid) using 142.4 mg (1.045 mmol) of zinc chloride and 4-tert-butylphenyl lithium (prepared by adding 100.6 mg (0.97 ml, 1.57 mmol) of tert-butyllithium (1.5M solution in pentane) to a cold solution (−78° C.) of 167.0 mg (0.78 mmol) of 4-tert-butylbromobenzene in 1.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.99 (2H, d, J=8.4 Hz), 7.55 (2H, d, J=8.4 Hz), 7.28–7.45 (7H, m), 6.02 (1H, t, J=4.9 Hz), 4.38 (2H, q, J=7.2 Hz), 2.36 (2H, d, J=4.9 Hz), 1.59 (3H, s), 1.40 (3H, t, J=7.2 Hz), 1.39 (9H, s), 1.35 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-chlorophenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 7)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 249.0 mg (1.827 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4-chlorophenyl lithium (prepared by adding 167.7 mg (1.54 ml, 2.62 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78°[°C.) of 252.4 mg (1.305 mmol) of 4-chloro-1-bromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.4 Hz), 7.53 (2H, d, J=8.4 Hz), 7.40–7.27 (6H, m), 7.12 (1H, d, J=1.6 Hz), 6.00 (1H, t, J=4.8 Hz), 4.37 (2H, q, J=7.1 Hz), 2.35 (2H, d, J=4.8. Hz), 1.40 (2H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methoxyphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 8)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 249.0 mg (1.827 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4-methoxyphenyl lithium (prepared by adding 167.7 mg (1.54 ml, 2.62 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 244.1 mg (1.305 mmol) of 4-methoxy-1-bromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.5 Hz), 7.52 (2H, d, J=8.6 Hz), 7.40–7.21 (5H, m), 6.95 (2H, d, J=8.7 Hz), 5.97 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 4.34 (3H, s), 2.34 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-trifluoromethylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 9)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenylethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 249.0 mg (1.827 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4-trifluoromethylphenyl lithium (prepared by adding 167.7 mg (1.54 ml, 2.62 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 296.6 mg (1.305 mmol) of 4-trifluoromethylbromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.5 Hz), 7.67 (2H, d, J=8.3 Hz), 7.54–7.36 (6H, m), 7.10 (1H, d, J=1.6 Hz), 6.06 (1H, t, J=4.8 Hz), 4.37 (2H, q, J=7.1 Hz), 2.38 (2H, d, J=4.8 Hz), 1.39 (3H, t, J=7.1 Hz), 1.35 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-pyridyl)-2-naphthalenyl)ethynyl]benzoate (Compound 10)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.52 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 142.4 mg (1.045 mmol) of zinc chloride and 2-lithiopyridine (prepared by the addition of 100.6 mg (0.97 ml, 1.57 mmol) of tert-butyllithium (1.5M solution in pentane) to a cold solution (−78° C.) of 123.8 mg (0.784 mmol) of 2-bromopyridine in 1.0 ml of THF). 1H NMR (d6-acetone): [δ 8.64 (1H, m), 7.99 (2H, d, J=8.5 Hz), 7.85 (1H, ddd, J=1.8, 7.7, 9.5 Hz), 7.58 (2H, d, J=8.4 Hz), 7.50 (1H, d, J=7.7 Hz), 7.47 (2 H, d, J=1.1 Hz), 7.35 (2H, m), 6.32 (1H, t, J=4.8 Hz), 4.34 (2H, q, J=7.2 Hz), 2.42 (2H, d, J=7.4 Hz), 1.35 (3H, t, J=7.0 hz), 1.35 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-pyridyl-2-naphthalenyl)ethynyl]benzoate (Compound 11)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenylethynyl]benzoate (Compound 1), 170.0 mg (0.35 mmol) of ethyl 4-[(5,6-dihydro-5,5dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 142.4 mg (1.045 mmol) of zinc chloride and 3-lithiopyridine (prepared by the addition of 100.2 mg (0.92 ml, 1.56 mmol) of tert-butyllithium (1.5M solution in pentane) to a cold solution (−78° C.) of 123.8 mg (0.784 mmol) of 3-bromopyridine in 1.0 ml of THF). 1H NMR ($CDCl_3$): δ 8.63–8.61 (2H, dd, J=1.7 Hz), 7.99 2H, d, J=8.4 Hz), 7.67 (1H, dt, J=7.9 Hz), 7.52 (2H, d, J=8.4 Hz), 7.43–7.34 (3H, m), 7.10 (1H, d, J=1.6 Hz), 6.07 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.40 (2H, d, J=4.7 Hz), 1.390 (3H, t, J=7.1 Hz), 1.36 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-methyl-5-pyridyl)-2-naphthalenyl)ethynyl]benzoate (Compound 12)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenlyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.522 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 142.4 mg (1.045 mmol) of zinc chloride and 2-methyl-5-lithiopyridine (prepared by the addition of 100.5 mg (0.92 ml, 1.57 mmol) of tert-butyllithium (1.7 M solution in pentane) to a cold solution (−78° C.) of 134.8 mg (0.784 mmol) of 2-methyl-5-bromopyridine in 1.0 ml of THF). 1H NMR ($CDCl_3$): δ 8.50 (1H, d, J=2.2 Hz), 7.99 (2H, d, J=8.3 Hz), 7.56 (1H, dd, J=2.3, 8.0 Hz), 7.53 (2H, d, J=8.4 Hz), 7.43 (1H, dd, J=2.3, 8.0 Hz), 7.37 (2H, d, J=8.0 Hz), 7.21 (1H, d, J=8.1 Hz), 7.11 (1H, d, J=1.5 Hz), 6.04 (1H, t, J=4.7 Hz), 4.38 (2H, q, J=7.2 Hz), 2.63 (3H, s), 2.38 (2H, d, J=4.6 Hz), 1.40 (3H, t, J=7.1 Hz), 1.35 (6H, s).

Ethyl 4-[(5,6-hydro-5,5-dimethyl-8-(3-((2,2-dimethylethyl)-dimethylsiloxy)phenyl)-2-naphthalenyl) ethynyl]benzoate (Compound H)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound G), 150.0 mg (0.314 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 150.0 mg (1.10 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 3-((2,2-dimethylethyl)dimethylsiloxy)phenyl lithium (prepared by adding 100.2 mg (0.92 ml, 1.564 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 226.0 mg (0.787 mmol) of 3-((2,2-dimethylethyl)dimethylsiloxy)bromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.4 Hz), 7.51 (2H, d, J=8.4 Hz), 7.40–7.22 (4H, m), 6.95 (1H, d, J=7.6 Hz), 6.84–6.82 (2H, m), 6.00 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.35 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (3H, s), 0.99 (9H, s), 0.23 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-((2,2-dimethylethyl)-dimethylsiloxy)phenyl)-2-naphthalenyl) ethynyl]benzoate (Compound I)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 210.0 mg (0.439 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 209.0 mg (1.53 mmol) of zinc chloride, 24 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4-((2,2-dimethylethyl)dimethylsiloxy)phenyl lithium (prepared by adding 140.3 mg (1.30 ml, 2.19 mmol) of tert-butyllithium (1.7M solution in pentane) to a cold solution (−78° C.) of 315.0 mg (1.09 mmol) of 4-((2,2-dimethylethyl)dimethylsiloxy)bromobenzene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.4 Hz), 7.51 (2H, d, J=8.4 Hz), 7.39–7.25 (3H, m), 7.21 (2H, d, J=8.5 Hz), 5.87 (2H, d, J=8.5 Hz), 5.96 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.33 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.33 (6H, s), 1.01 (9H, s), 0.25 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-hydroxyphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 13)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-((2,2-dimethylethyl)-dimethylsiloxy)-phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound H) 60.0 mg (0.114 mmol) in 1.0 ml of THF at room temperature was added 91.5 mg (0.35 ml, 0.35 mmol) of tetrabutylamonium flouride (1 M solution in THF). After stirring overnight, the solution was diluted with EtOAc and washed with H_2O and saturated aqueous NaCl, before being dried over $MgSO_4$. Removal of the solvents under reduced pressure, followed by column chromatography (4:1, Hexanes:EtOAc) afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=7.8 Hz), 7.52 (2H, d, J=8.3 Hz), 7.39–7.21 (4H, m), 6.93 (1H, d, J=7.5 Hz), 6.84 (1H, d, 7.1 Hz), 6.83 (1H, s), 6.01 1H, t, J=4.7 Hz), 4.91 (1H, s), 4.39 (2H, q, J=7.1 Hz), 2.35 (2H, d, J=4.7 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-hydroxyphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 14)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-((2,2-dimethylethyl)-dimethylsiloxy)phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound I) 50.0 mg (0.095 mmol) in 1.0 ml of THF at room temperature was added 73.2 mg (0.29 ml, 0.29 mmol) of tetrabutylamonium fluoride (1 M solution in THF). After stirring overnight, the solution was diluted with EtOAc and washed with H_2O and saturated aqueous NaCl, before being dried over $MgSO_4$. Removal of the solvents under reduced pressure, followed by column chromatography (4:1, Hexanes:EtOAc) afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 7.98 (2H, d, J=8.2 Hz), 7.52 (2H, d, J=8.3 Hz), 7.41–7.20 (5H, m), 6.88 (2H, d, J=8.4 Hz), 5.96 (1H, t, J=4.5 Hz), 4.37 (2H, q, J=7.1 Hz), 2.34 (2H, d, J=4.5 Hz), 1.39 (3H, t, J=7.1 Hz), 1.34 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(5-methylthiazol-2-yl)-2-naphthalenyl)ethynyl]benzoate (Compound 15)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 264.0 mg (0.552 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 150.0 mg (1.10 mmol) of zinc chloride, 14 mg (0.012 mmol) of tetrakis(triphenylphosphine)palladium(0) in 4.0 ml of THF, and 5-methylthiazol-2-yl lithium (prepared by adding 53.2 mg (0.53 ml, 0.83 mmol) of n-butyllithium (1.55 M solution in hexanes) to a cold solution (−78° C.) of 82.0 mg (0.83 mmol) of 5-methylthiazole in 5.0 ml of THF). 1H NMR ($CDCl_3$): δ 7.99 (2H, d, J=7.8 Hz), 7.88 (1H, d, J=1.5 Hz),

7.55 (2H, d, J=7.8 Hz), 7.54 (1H, s), 7.45 (1H, dd, J=1.5, 8.0 Hz), 7.35 (1H, d, J=7.9 Hz), 6.48 (1H, t, J=4.8 Hz), 4.38 (2H, q, J=7.1 Hz), 2.51 (3H, s), 2.38 (2H, d, J=4.8 Hz), 1.40 (3H, s), 1.32 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thiazolyl)-2-naphthalenyl)ethynyl]benzoate (Compound 15a)

A solution of 2-lithiothiazole was prepared by the addition of 41.2 mg (0.42 ml, 0.63 mmol) of n-butyl-lithium (1.5M solution in hexanes) to a cold solution (−78° C.) of 53.4 mg (0.63 mmol) of thiazole in 1.0 ml of THF. The solution was stirred at for 30 minutes and then a solution of 113.9 mg (0.84 mmol) of zinc chloride in 1.5 ml of THF was added. The resulting solution was warmed to room temperature, stirred for 30 minutes and then the organozinc was added via cannula to a solution of 200.0 mg (0.42 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl) oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) and 12.4 mg (0.01 mmol) of tetrakis(triphenylphosphine) palladium(0) in 1.5 ml of THF. The resulting solution was heated at 50° C. for 45 minutes, cooled to room temperature and diluted with sat. aqueous NH_4Cl. The mixture was extracted with EtOAc (40 ml) and the combined organic layers were washed with water and brine. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to a yellow oil. Purification by column chromatography (silica, 20% EtOAc-hexanes) yielded the title compound as a colorless oil. PMR ($CDCl_3$): δ 1.35 (6H, s), 1.40 (3H, t, J=7.1 Hz), 2.42 (2H, d, J=4.8 Hz), 4.38 (2H, q, J=7.1 Hz), 6.57 (1H, t, J=4.8 Hz), 7.33 (1H, d, J=3.3 Hz), 7.36 (1H, d, J=8.0 Hz), 7.46 (1H, dd, J=1.7 , 8.1 Hz), 7.55 (2H, d, J=8.4 Hz), 7.87 (1H, d, J=1.7 Hz), 7.92 (1H, d, J=3.3 Hz), 8.00 (2H, d, J=8.4 Hz).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylthiazol-2-yl)-2-naphthalenyl)ethynyl]benzoate (Compound 16)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenylethynyl]benzoate (Compound 1), 295.0 mg (0.617 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 168.0 mg (1.23 mmol) of zinc chloride, 16 mg (0.014 mmol) of tetrakis(triphenylphosphine)palladium(0) in 6.0 ml of THF, and 4-methylthiazol-2-yl lithium (prepared by adding 59.6 mg (0.60 ml, 0.93 mmol) of n-butyllithium (1.55 M solution in hexanes) to a cold solution (−78° C.) of 92.0 mg (0.93 mmol) of 4-methylthiazole in 6.0 ml of THF). 1H NMR ($CDCl_3$): δ 8.00 (2H, d, J=8.4 Hz), 7.80 (1H, d, J=1.7 Hz), 7.55 (2H, d, J=8.4 Hz), 7.45 (1H, dd, J=1.7, 8.0 Hz), 7.35 (1H, d, J=8.0 Hz), 6.87 (1H, s), 6.52 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.2 Hz), 2.54 (3H, s), 2.39 (2H, d, J=4.7 Hz), 1.40 (3H, t, J=7.2 Hz), 1.33 (3H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4,5-dimethylthiazol-2-yl)-2-naphthalenyl) ethynyl]benzoate (Compound 17)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 200.0 mg (0.418 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 110.0 mg (0.84 mmol) of zinc chloride, 12 mg (0.011 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 4,5-dimethylthiazol-2-yl lithium (prepared by adding 40.2 mg (0.39 ml, 0.63 mmol) of n-butyllithium (1.55 M solution in hexanes) to a cold solution (−78° C.) of 71.0 mg (0.63 mmol) of 4,5-dimethylthiazole in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 8.00 (2H, d, J=8.4 Hz), 7.82 (1H, d, J=1.7 Hz), 7.54 (2H, d, J=8.4 Hz), 7.43 (1H, dd, J=1.7, 8.0 Hz), 7.33 91H, d, J=8.0 Hz), 6.45 (1H, t, J=4.9 Hz), 4.38 (2H, q, J=7.1 Hz), 2.41 (3H, s), 2.40 (3H, s), 2.37 (2H, d, J=4.9 Hz), 1.40 (3H, t, J=7.1 Hz), 1.32 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(2-methyl-5-pyridyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 18)

A solution of 81.7 mg (0.194 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-methyl-5-pyridyl)-2-naphthalenyl)ethynyl]benzoate (Compound 12) and 40.7 mg (0.969 mmol) of $LiOH-H_2O$ in 3 ml of THF/water (3:1, v/v), was stirred overnight at room temperature. The reaction was quenched by the addition of saturated aqueous NH_4Cl and extracted with EtOAc. The combined organic layers were washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo to give the title compound as a colorless solid. 1H NMR (d6-DMSO): δ 8.41 (1H, d, J=1.9 Hz), 7.90 (2H, d, J=8.3 Hz), 7.63 (1H, dd, J=2.3, 7.9 Hz), 7.59 (2H, d, J=8.3 Hz), 7.49 (2H, m), 7.33 (1H, d, J=7.8 Hz), 6.95 (1H, s), 6.11 (1H, t, J=4.5 Hz), 2.52 (3H, s), 2.37 (2H, d, J=4.6 Hz), 1.31 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(2-pyridyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 19)

A solution of 80.0 mg (0.196 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-pyridyl)-2-naphthalenyl) ethynyl]benzoate (Compound 10) and 20.6 mg (0.491 mmol) of $LiOH-H_2O$ in 3 ml of THF/water (3:1, v/v), was stirred overnight at room temperature. The reaction was quenched by the addition of saturated aqueous NH_4Cl and extracted with EtOAc. The combined organic layers were washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo to give the title compound as a colorless solid. 1H NMR (d6-DMSO): δ 8.64 (1H, m), 7.94 (2H, d, J=8.3 Hz), 7.87 (1H, dt, J=1.7, 7.8 Hz), 7.58 (2H, d, J=8.3 Hz), 7.50 (1H, d, J=8.2 Hz), 7.47 (2H, s), 7.37 (1H, m), 7.25 (1H, s), 6.30 (1H, t, J=4.6 Hz), 2.39 (2H, d, J=4.6 Hz), 1.31 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(3-methylphenyl)-2-naphthalenyl)ethynl]benzoic acid (Compound 20)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 2) 30.0 mg (0.071 mmol) in 3 ml of EtOH and 2 ml of THF was added 28.0 mg (0.70 mmol, 0.7 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ0 7.90 (2H, d, J=8.5 Hz), 7.59 (2H, d, J=8.5 Hz), 7.46 (2H, s), 7.32–7.13 (4H, m), 7.10 (1H, s), 6.98 (1H, t, J=4.5 Hz), 2.34 (3H, s), 2.31 (2H, d, J=4.5 Hz), 1.30 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-ethylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 21)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-ethylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 5) 47.0 mg (0.108 mmol) in 3 ml of EtOH and 2 ml of THF was added 28.0 mg (0.70 mmol, 0.7 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.90 (2H, d, J=8.3 Hz), 7.59 (2H, d, J=8.3 Hz), 7.46 (2H, s), 7.29–7.21 (4H, m), 7.02 (1H, s), 6.01 (1H, t, J=4.5 Hz), 2.64 (2H, q, J=7.5 Hz), 2.33 (2H, d, J=4.5 Hz), 1.29 (6H, s), 1.22 (3H, t, J=7.5 Hz).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-methoxyphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 22)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methoxyphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 8) 80.0 mg (0.183 mmol) in 3 ml of EtOH and 2 ml of THF was added 40.0 mg (1.00 mmol, 1.0 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.90 (2H, d, J=8.3 Hz), 7.60 (2H, d, J=8.3 Hz), 7.45 (2H, s), 7.24 (2H, d, J=8.6 Hz), 7.02–6.89 (3H, m), 5.98 (1H, t, J=4.4 Hz), 3.79 (3H, s), 2.31 (2H, d, J=4.7 Hz), 1.29 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-trifluoromethylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 23)

To a solution of ethyl 4-[(5,6-dihydro-5,5-diethyl-8-(4-trifluoromethylphenyl)-2-naphthalenylethynyl]benzoate (Compound 9) 70.0 mg (0.148 mmol) in 3 ml of EtOH and 2 ml of THF was added 60.0 mg (1.50 mmol, 1.50 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.90 (2H, d, J=8.3 Hz), 7.80 (2H, d, J=8.1 Hz), 7.61–7.47 (6H, m), 6.97 (2H, s), 6.16 (1H, t, J=4.5 Hz), 2.37 (2H, d, J=4.6 Hz), 1.30 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(3,5-dimethylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 24)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3,5-dimethylphenyl)-2-naphthalenyl)ethynyl]-benzoate (Compound 4) 90.0 mg (0.207 mmol) in 3 ml of EtOH and 2 ml of THF was added 48.0 mg (1.20 mmol, 1.20 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.90 (2H, d, J=8.2 Hz), 7.59 (2H, d, J=8.2 Hz), 7.45 (2H, s), 7.00 (1H, s), 6.97 (1H, s), 5.97 (1H, t, J=4.5 Hz), 2.31 (2H, d, J=4.5 Hz), 2.30 (6H, s), 1.29 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-chlorophenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 25)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-chlorophenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 7) 80.0 mg (0.181 mmol) in 3 ml of EtOH and 2 ml of THF was added 48.0 mg (1.20 mmol, 1.20 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.90 (2H, d, J=8.3 Hz), 7.60 (2H, d, J=8.3 Hz), 7.51–7.48 (4H, m), 7.34 (2H, d, J=8.4 Hz), 6.97 (1H, s), 6.07 (1H, t, J=4.5 Hz), 2.34 (2H, d, J=4.6 Hz), 1.29 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(3-pyridyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 26)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-pyridyl)-2-naphthalenyl)ethynyl]benzoate (Compound 11) 45.0 mg (0.110 mmol) in 3 ml of EtOH and 2 ml of THF was added 48.0 mg (1.20 mmol, 1.20 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 8.60 (1H, d, J=4.6 Hz), 8.55 (1H, s), 7.90 (2H, d, J=8.3 Hz), 7.76 (1H, d, J=7.5 Hz), 7.60 (2H, d, J=8.3 Hz), 7.51–7.46 (3H, m), 6.94 (1H, s), 6.14 (1H, t, J=4.5 Hz), 2.37 (2H, d, J=4.5 Hz), 1.31 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(2-methylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 27)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-methylphenyl)-2-naphthalenyl)ethynyl]-benzoate (Compound 3) 80.0 mg (0.190 mmol) in 3 ml of EtOH and 2 ml of THF was added 60.0 mg (1.50 mmol, 1.50 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (DMSO): δ 7.89(2H, d, J=8.4 Hz), 7.57 (2H, d, J=8.4 Hz), 7.46 (2H, s), 7.29–7.14 (4H, m), 6.59 (1H, s), 5.90 (1H, t, J=4.7 Hz), 2.39 (2H, m), 2.60 (3H, s), 1.39 (3H, s), 1.29 (3H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(3-hydroxyphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 28)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(3-((2,2-dimethylethyl)-dimethylsiloxy)phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound H) 40.0 mg (0.076 mmol) in 3 ml of EtOH and 2 ml of THF was added 40.0 mg (1.00 mmol, 1.00 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (d6-acetone): δ 7.90 (2H, d, J=8.3 Hz), 7.49 (2H, d, J=8.4 Hz), 7.35 (2H, s), 7.15–7.07 (2H, m), 6.77–6.69 (3H, m), 5.92 (1H, t, J=4.7 Hz), 2.25 (2H, d, J=4.7 Hz), 1.23 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-hydroxyphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 29)

To a solution of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-((2,2-dimethylethyl)-dimethylsiloxy)phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1) 75.0 mg (0.143 mmol) in 3 ml of EtOH and 2 ml of THF was added 60.0 mg (1.50 mmol, 1.50 ml) of NaOH (1.0 M aqueous solution). The solution was heated to 50° C. for 2 hours, cooled to room temperature, and acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (d6-acetone): δ 8.01 (2H, d, J=8.3 Hz), 7.59 (2H, d, J=8.4 Hz), 7.45 (2H, s), 7.20–7.17 (3H, m), 6.92–6.89 (2H, m), 5.97 (1H, t, J=4.7 Hz), 2.35 (2H, d, J=4.7 Hz), 1.34 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl-8-(5-methylthiazol-2-yl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 30)

To a solution of ethyl 4-[5,6-dihydro-5,5-dimethyl-8-(5-methylthiazol-2-yl)-2-naphthalenyl]ethynylbenzoate (Compound 15) (100 mg, 0.23 mmol) and 4 ml of EtOH at room temperature was added aqueous NaOH (1 ml, 1 M, 1 mmol). The resulting solution was warmed to 50° C. for 1 hour and concentrated in vacuo. The residue was suspended in a solution of CH_2Cl_2 and ether (5:1) and acidified to pH 5 with 1M aqueous HCl. The layers were separated and the organic layer was washed with brine, dried (Na_2SO_4), filtered and the solvents removed under reduced pressure to give the title compound as a white solid. 1H NMR (d6-DMSO): δ 7.96 (1H, d, J=1.7 Hz), 7.95 (2H, d, J=8.0 Hz), 7.65 (2H, d, J=8.0 Hz), 7.64 (1H, s), 7.53 (1H, dd, J=1.7, 8.0 Hz), 7.46 (1H, d, J=8.0 Hz), 6.59 (1H, t, J=4.5 Hz), 2.50 (3H, s), 2.39 (2H, d, J=4.5 Hz), 1.27 (6H, s).

4-[(5,6-dihydro-5,5-dimethyl-8-(2-thiazolyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 30a)

A solution of 33.9 mg (0.08 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thiazolyl)-2-naphthalenyl) ethynyl]benzoate (Compound 15a) and 8.5 mg (0.20 mmol) of LiOH-H_2O in 3 ml of THF/water (3:1, v/v), was stirred overnight at room temperature. The reaction was quenched by the addition of sat. aqueous NH_4Cl and extracted with EtOAc. The combined organic layers were washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo to give the title compound as a colorless solid. PMR (d6-DMSO): δ 1.29 (6H, s), 2.42 (2H, d, J=4.6 Hz), 6.68 (1H, t, J=4.6 Hz), 7.51 (2H, m), 7.62 (2H, d, J=8.2 Hz), 7.77 (1H, d, J=3.3 Hz), 7.93 (2H, d, J=8.2 Hz), 7.98 (1H, d, J=3.3 Hz).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-methylthiazol-2-yl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 31)

To a solution of ethyl 4-[5,6-dihydro-5,5-dimethyl-8-(4-methylthiazol-2-yl)-2-naphthalenyl]ethynylbenzoate (Compound 16) (145.0 mg, 0.34 mmol) and 4 ml of EtOH at room temperature was added aqueous NaOH (1 ml, 1 M, 1 mmol). The resulting solution was warmed to 50° C. for 1 hour and concentrated in vacuo. The residue was suspended in a solution of CH_2Cl_2 and ether (5:1) and acidified to pH 5 with 1M aqueous HCl. The layers were separated and the organic layer was washed with brine, dried (Na_2SO_4), filtered and the solvents removed under reduced pressure to give the title compound as a white solid. 1H NMR (d6-DMSO): δ 7.94 (2H, d, J=8.1 Hz), 7.87 (1H, d, J=1.6 Hz), 7.63 (2H, d, J=8.3 Hz), 7.50 (1H, dd, J=1.6, 8.1 Hz), 7.45 (1H, d, J=8.1 Hz), 7.27 (1H, s), 6.58 (1H, t, J=4.8 Hz), 2.43 (3H, s), 2.37 (2H, d, J=4.8 Hz), 1.26 (6H, s).

4-[(5,6-Dihydro-5,5-dimethyl -8-(4,5-dimethylthiazol-2-yl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 32)

To a solution of ethyl 4-[5,6-dihydro-5,5-dimethyl-8-(4,5-dimethylthiazol-2-yl)-2-naphthalenyl]ethynylbenzoate (Compound 17) (58.0 mg, 0.13 mmol) and 4 ml of EtOH at room temperature was added aqueous NaOH (1 ml, 1 M, 1 mmol). The resulting solution was warmed to 50° C. for 1 hour and concentrated in vacuo. The residue was suspended in a solution of CH_2Cl_2 and ether (5:1) and acidified to pH 5 with 1M aqueous HCl. The layers were separated and the organic layer was washed with brine, dried (Na_2SO_4), filtered and the solvents removed under reduced pressure to give the title compound as a white solid. 1H NMR (d6-DMSO): δ 7.94 (2H, d, J=8.4 Hz), 7.86 (1H, d, J=1.6 Hz), 7.61 (2H, d, J=8.3 Hz), 7.50 (1H, dd, J=1.6, 8.0 Hz), 7.45 (1H, d, J=8.0 Hz), 6.51 (1H, t, J=4.9 Hz), 2.37 (3H, s), 2.36 (2H, d, J=4.6 Hz), 2.32 (3H, s), 1.26 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(5-methyl-2-thienyl)-2-naphthalenyl)ethynyl]benzoate (Compound 33)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 170.0 mg (0.366 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 202.0 mg (1.48 mmol) of zinc chloride, 24 mg (0.022 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF, and 5-methyl-2-lithiothiophene (prepared by adding 58.6 mg (0.36 ml, 0.915 mmol) of n-butyllithium (2.5 M solution in hexanes) to a cold solution (−78° C.) of 89.8 mg (0.915 mmol) of 2-methylthiophene in 2.0 ml of THF). 1H NMR ($CDCl_3$): δ 8.00 (2H, d, J=8.3 Hz), 7.59 (1H, d, J=1.7 Hz), 7.55 (2H, d, J=8.2 Hz), 7.43 (1H, dd, J=1.7, 8.0 Hz), 7.35 (1H, d, J=8.0 Hz), 6.87 (1H, d, J=3.5 Hz), 6.74 (1H, d, J=2.8 Hz), 6.15 (1H, t, J=4.8 Hz), 4.38 (2H, q, J=7.1 Hz), 2.52 (3H, s), 2.32 (2H, d, J=4.8 Hz). 1.40 (3H, t, 7.1 Hz), 1.32 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thienyl)-2-naphthalenyl)ethynyl]benzoate (Compound 33a)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.52 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 186.8 mg (1.37 mmol) of zinc chloride 37.1 mg (0.03 mmol) of tetrakis(triphenylphosphine)palladium(0) and 2-lithiothiophene (prepared by the addition of 65.9 mg (0.69 ml, 1.03 mmol) of n-butyllithium (1.5M solution in hexane) to a cold solution (−78° C.) of 86.5 mg (1.03 mmol) of thiophene in 1.0 ml of THF). PMR ($CDCl_3$): δ 1.33 (6H, s), 1.36 (3H, t, J=7.1 Hz), 2.38 (2H, d, J=4.7 Hz), 4.34 (2H, q, J=7.2 Hz), 6.25 (1H, t, J=4.7 Hz), 7.13 (2H, m), 7.47 (4H, m), 7.62 (2H, d, J=8.5 Hz), 8.00 (2H, d, J=8.5 Hz).

4-[(5,6-Dihydro-5,5-dimethyl-8-(5-methyl-2-thienyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 34)

To a solution of ethyl 4-[5,6-dihydro-5,5-dimethyl-8-(5-methyl-2-thienyl)-2-naphthalenyl]ethynylbenzoate (Compound 33) (35.0 mg, 0.082 mmol) in 2 ml of EtOH and 1 ml THF at room temperature was added aqueous NaOH (1 ml, 1 M, 1 mmol). The resulting solution was stirred at room temperature overnight and then acidified with 10% HCl. Extraction with EtOAc, followed by drying over Na_2SO_4, and removal of the solvents under reduced pressure afforded the title compound as a colorless solid. 1H NMR (d6-acetone): δ 8.03 (2H, d, J=8.6 Hz), 7.63 (2H, d, J=8.6 Hz), 7.54–7.48 (3H, m), 6.89 (1H, m), 6.18 (1H, t, J=4.7 Hz), 2.49 (3H, s), 2.35 (2H, d, J=4.7 Hz), 1.32 (6H, s).

4-[(5,6-dihydro-5,5-dimethyl-8-(2-thienyl)-2-naphthalenyl) ethynyl]benzoic acid (Compound 34a)

Employing the same general procedure as for the preparation of 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thiazolyl)-2-naphthalenyl)ethynylbenzoic acid (Compound 30a), 70.0 mg (0.17 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-thienyl)-2-naphthalenyl)ethynyl]benzoate (Compound 33a) was converted into the title compound (colorless solid) using 17.8 mg (0.42 mmol) of LiOH in H_2O. PMR (d_6-DMSO): δ 1.27 (6H, s), 2.33 (2H, d, J=4.9 Hz), 6.23 (1H, t, J=4.9 Hz), 7.14 (2H, m), 7.38–7.56 (4H, m), 7.61 (2H, d, J=8.3 Hz), 7.92 (2H, d, J=8.3 Hz).

5,6-Dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenecarboxylic acid (Compound K)

A solution of 3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-7-bromonaphthalene (Compound D) (250.0 mg, 0.764 mmol) in 2.0 ml of THF was cooled to −78° C. and 1.0 ml of t-butyllithium (1.68 mmol, 1.7 M solution in pentane) was added slowly. After stirring for 1 hour at −78° C. gaseous CO_2 (generated by evaporation of Dry-Ice, and passed though a drying tube) was bubbled through the reaction for 1 hour. The solution was then allowed to warm to room temperature and the reaction was quenched by the addition of 10% HCl. Extraction with EtOAc was followed by washing the combined organic layers with H_2O and saturated aqueous NaCl, and drying over $MgSO_4$. Removal of the solvents under reduced pressure and washing of the solid with hexanes afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 7.94 (1H, dd, J=1.8, 8.1 Hz), 7.76 (1H, d, J=1.8 Hz), 7.45 (1H, d, J=8.1 Hz), 7.24 (4H, m), 6.01 (1H, t, J=4.7 Hz), 2.40 (3H, s), 2.36 (2H, d, J=4.7 Hz), 1.35 (6H, s).

Ethyl 4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoate (Compound 35)

A solution of 170.0 mg (0.58 mmol) 5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenecarboxylic acid (Compound K) 115.0 mg (0.70 mmol) of ethyl 4-aminobenzoate, 145.0 mg (0.76 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and 92.4 mg (0.76 mmol) of 4-dimethylaminopyridine in 6.0 ml of DMF was stirred overnight at room temperature. Ethyl acetate was added and the resulting solution washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was isolated as a colorless solid by column chromatography (10 to 15% EtOAc/hexanes). 1H NMR ($CDCl_3$): δ 8.02 (2H, d, J=8.7 Hz), 7.72 (2H, m), 7.65 (2H, d, J=8.7 Hz), 7.52 (1H, d, J=1.8 Hz), 7.48 (1H, d, J=8.0 Hz), 7.25 (4H, m), 6.15 (1H, t, J=4.9 Hz), 4.36 (2H, q, J=7.1 Hz), 2.40 (3H, s), 2.38 (2H, d, J=4.9 Hz), 1.39 (3H, t, J=7.1 Hz), 1.37 (6H, s).

4-[[(5,6-Dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoic acid (Compound 36)

To a solution of 26.5 mg (0.06 mmol) ethyl 4[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl) carbonyl]amino]-benzoate (Compound 35) in 3.0 ml EtOH and 4.0 ml of THF was added 240.1 mg NaOH (6.00 mmol, 3.0 ml of a 2M aqueous solution). After stirring at room temperature for 72 hours, the reaction was quenched by the addition of 10% HCl. Extraction with EtOAc, and drying of the organic layers over $MgSO_4$, provided a solid after removal of the solvent under reduced pressure. Crystallization from CH_3CN afforded the title compound as a colorless solid. 1H NMR (d6-DMSO): δ 10.4 (1H, s), 7.91–7.81 (5H, m), 7.54 (1H, d, J=8.1 Hz), 7.45 (1H, d, J=1.7 Hz), 7.23 (4H, s), 6.04 (1H, t, J=4.7 Hz), 2.35 (5H, s), 1.33 (6H, s).

Ethyl 4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methyl-phenyl)-2-naphthalenyl)carbonyl]oxy]-benzoate (Compound 37)

A solution of 25.0 mg (0.086 mmol) 5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenecarboxylic acid (Compound K) 17.5 mg (0.103 mmol) of ethyl 4-hydroxybenzoate, 21.4 mg (0.112 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and 12.6 mg (0.103 mmol) of 4-dimethylaminopyridine in 2.0 ml of DMF was stirred overnight at room temperature. Ethyl acetate was added and the resulting solution washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, before being dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was isolated by column chromatography as a pale-yellow solid (10% EtOAc/hexanes). 1H NMR ($CDCl_3$): δ 8.08 (2H, d, J=8.1 Hz), 8.05 (1H, dd, J=1.8, 8.1 Hz), 7.89 (1H, d, J=1.8 Hz), 7.50 (2H, d, J=8.1 Hz), 7.22 (5H, m), 6.05 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.39 (2H, d, J=4.7 Hz), 2.38 (3H, s), 1.39 (3H, t, J=7.1 Hz), 1.37 (6H, s).

2-Trimethylsilylethyl 4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]oxy]-benzoate (Compound 38)

A solution of 93.5 mg (0.320 mmol) 5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenecarboxylic acid (Compound K) 76.0 mg (0.319 mmol) of 2-trimethylsilylethyl-4-hydroxybenzoate, 80.0 mg (0.417 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and 51.0 mg (0.417 mmol) of 4-dimethylaminopyridine in 4.0 ml of DMF was stirred overnight at room temperature. Ethyl acetate was added and the resulting solution washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, before being dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was isolated as a colorless solid by column chromatography (5% EtOAc/hexanes). 1H NMR ($CDCl_3$): δ 8.08 (2H, d, J=8.8 Hz), 8.05 (1H, dd, J=1.8, 8.1 Hz), 7.50 (1H, d, J=8.1 Hz), 7.26–7.18 (6H, m), 6.05 (1H, t, J=4,7 Hz), 4.42 (2H, t, J=8.4 Hz), 2.40 (2H, d, J=4.7 Hz), 2.39 (3H, s), 1.38 (6H, s), 0.09 (9H, s).

4-[[(5,6-Dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]oxy]-benzoic acid (Compound 39)

A solution of 110.0 mg (0.213 mmol) 2-trimethylsilylethyl 4[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]oxy]-benzoate (Compound 38) and 167.3 mg of tetrabutylammonium flouride (0.640 mmol, 0.64 ml of a 1M solution in THF) in 2.0 ml THF was stirred at room temperature for 22 hours. Ethyl acetate was added and the resulting solution washed with H_2O and saturated aqueous NaCl then dried over $MgSO_4$. Removal of the solvents under reduced pressure and washing of the residual solid with EtOAc and CH_3CN afforded the title compound as a colorless solid. 1H NMR (d6-acetone): δ 8.10 (2H, d, J=8.8 Hz), 8.06 (1H, dd, J=2.0, 8.1 Hz), 7.82 (1H, d, J=1.9 Hz), 7.64 (1H, d, J=8.1 Hz), 7.35 (2H, d, J=8.6 Hz), 7.25 (4H, m), 6.08 (1H, t, J=4.7 Hz), 2.42 (2H, d, J=4.7 Hz), 2.35 (3H, s), 1.39 (6H, s).

Ethyl 2-fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoate (Compound 40)

A solution of 115.0 mg (0.41 mmol) 5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenecarboxylic acid (Compound K) 89.0 mg (0.49 mmol) of ethyl 2-fluoro-4-aminobenzoate, 102.0 mg (0.53 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, and 65.0 mg (0.53 mmol) of 4-dimethylaminopyridine in 5.0 ml of DMF was stirred at 50° C. for 1 hour and then overnight at room temperature. Ethyl acetate was added and the resulting solution washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, before being dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was isolated as a colorless solid by column chromatography (20% EtOAc/hexanes). 1H NMR ($CDCl_3$): δ 7.96 (1H, s), 7.89 (1H, t, J=8.4 Hz), 7.70 (2H, m), 7.52 (1H, d, J=1.9 Hz), 7.45 (1H, d, J=8.1 Hz), 7.23 (5H, m), 6.04 (1H, t, J=4.8 Hz), 4.36 (2H, q, J=7.1 Hz), 2.38 (3H, s), 2.35 (2H, d, J=4.8 Hz), 1.39 (3H, t, J=7.1 Hz), 1.36 (6H, s).

2-Fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoic acid (Compound 41)

To a solution of 41.6 mg (0.091 mmol) ethyl 2-fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoate (Compound 40) in 2.0 ml EtOH and 2.0 ml of THF was added 40.0 mg NaOH (1.00 mmol, 1.0 ml of a 1 M aqueous solution). After stirring at room temperature for overnight, the reaction was quenched by the addition of 10% HCl. Extraction with EtOAc, and drying of the organic layers over $MgSO_4$, provided a solid after removal of the solvent under reduced pressure. Crystallization from CH_3CN afforded the title compound as a pale-yellow solid. 1H NMR (d6-acetone): δ 9.84 (1H, s), 7.94–7.83 (3H, m), 7.64 (1H, dd, J=2.0 Hz), 7.53 (2H, d, J=8.1 Hz), 7.23 (4H, s), 6.04 (1H, t, J=4.7 Hz), 2.38 (2H, d, J=4.7 Hz), 2.36 (3H, s), 1.35 (6H, s).

Ethyl 4[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)thiocarbonyl]amino]-benzoate (Compound 42)

A solution of 110.0 mg (0.25 mmol) ethyl 4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl) carbonyl]amino]-benzoate (Compound 35) and 121.0 mg

(0.30 mmol) of [2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide] (Lawesson's Reagent) in 12.0 ml of benzene was refluxed overnight. Upon cooling to room temperature, the mixture was filtered and the filtrate concentrated under reduced pressure. The title compound was isolated by column chromatography (10 to 25% EtOAc/hexanes) as a yellow solid. 1H NMR ($CDCl_3$): δ 8.92 (1H, s), 8.06 (2H, t, J=8.5 Hz), 7.88–7.70 (3H, m), 7.42 (2H, d, J=8.1 Hz), 7.18 (4H, m), 6.03 (1H, t, J=4.7 Hz), 4.37 (2H, q, J=7.1 Hz), 2.38 (3H, s), 2.36 (2H, d, J=4.7 Hz), 1.56 (3H, t, J=7.1 Hz), 1.35 (6H, s).

4-[[(5,6-Dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)thiocarbonyl]amino]-benzoic acid (Compound 43)

To a solution of 84.0 mg (0.184 mmol) ethyl 4[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl) thiocarbonyl]amino]-benzoate (Compound 42) in 2.0 ml EtOH and 2.0 ml of THF was added 60.0 mg NaOH (1.50 mmol, 1.5 ml of a 1 M aqueous solution). After stirring at room temperature overnight, the reaction was quenched by the addition of 10% HCl. Extraction with EtOAc, and drying of the organic layers over $MgSO_4$, provided a solid after removal of the solvent under reduced pressure. Crystallization from CH_3CN afforded the title compound as a yellow solid. 1H NMR (d6-acetone): δ 10.96 (1H, s), 8.05 (4H, m), 7.72 (1H, dd, J=2.0, 8.0 Hz), 7.54 (1H, s), 7.46 (1H, d, J=8.1 Hz), 7.20 (4H, m), 6.04 (1H, t, J=4.7 Hz), 2.38 (2H, d, J=4.7 Hz), 2.33 (3H, s), 1.35 (6H, s).

2-acetyl-6-bromonaphthalene (Compound L)

To a cold (10° C.) mixture of 44.0 g (0.212 mol) of 2-bromonaphthalene and 34.0 g (0.255 mol) of aluminum chloride in 400 ml of nitrobenzene was added 21.0 g (267 mmol) of acetyl chloride. The mechanically stirred reaction mixture was warmed to room temperature, and heated to 40° C. for 18 hours. After cooling to 0° C. in an ice bath, the reaction was quenched by the addition of 12M HCl (70 ml). The layers were separated and the organic phase was washed with water and dilute aqueous Na_2CO_3. Kugelrohr distillation, followed by recrystallization from 10% EtOAc-hexane yielded 23 g of the title compound as a tan solid. 1H NMR ($CDCl_3$): δ 8.44 (1H, br s), 8.04–8.10 (2H, m), 7.85 (1H, d, J=8.5 Hz), 7.82 (1H, d, J=8.8 Hz), 7.64 (1H, d, J=8.8 Hz), 2.73 (3H, s).

6-bromo-2-naphthalenecarboxylic acid (Compound M)

To a solution of sodium hypochlorite (62 ml, 5.25% in water (w/w), 3.6 g, 48.18 mmol) and sodium hydroxide (6.4 g, 160.6 mmol) in 50 ml of water was added a solution of 2-acetyl-6-bromonaphthalene (Compound L) 4 g, (16.06 mmol) in 50 ml of 1,4-dioxane. The yellow solution was heated to 70° C. in an oil bath for 2 hours, cooled to ambient temperature, and extracted with ethyl ether (2×50 ml). The aqueous layers were diluted with $NaHSO_3$ solution (until KI indicator solution remained colorless) and then acidified (pH<2) with 1N sulfuric acid to give a white precipitate. The mixture was extracted with ethyl ether, and the combined organic phase washed with saturated aqueous NaCl, dried ($MgSO_4$) and concentrated to give 3.54 g (88%) of the title compound as a solid. 1H NMR (DMSO-d6): δ 8.63 (1H, br s), 8.32 (1H, d, J=2.0 Hz), 8.10 (1H, d, J=8.8 Hz), 8.00–8.05 (2H,,m), 7.74 (1H, dd, J=2.0, 8.8 Hz).

Ethyl 6-bromo-2-naphthalenecarboxylate (Compound N)

To a solution of 6-bromo-2-naphthalenecarboxylic acid (Compound M) 3.1 g, (12.43 mmol) in ethanol (30 ml, 23.55 g, 511.0 mmol) was added 18M sulfuric acid (2 ml). The solution was refluxed for 30 minutes, cooled to room temperature, and the reaction mixture partitioned between pentane (100 ml) and water (100 ml). The aqueous phase was extracted with pentane (100 ml) and the combined organic layers washed with saturated aqueous NaCl (100 ml), dried ($MgSO_4$), and concentrated to yield an off-white solid. Purification by flash chromatography (silica, 10% EtOAc-hexane) afforded the title compound as a white solid. 1H NMR ($CDCl_3$): δ 8.58 (1H, br s), 8.10 (1H, dd, J=1.7, 9 Hz), 8.06 (1H, d, J=2 Hz), 7.83 (1H, d, J=9 Hz), 7.80 (1H, d, J=9 Hz), 7.62 (1H, dd, J=2, 9 Hz).

Ethyl (E)4-[2-(5,6,7,8-tetrahydro-5,5-dimethyl-8-oxo-2-naphthalenyl]ethenyl]-benzoate (Compound O)

To a solution of 520.0 mg (2.00 mmol) of 3,4-dihydro-4,4-dimethyl-7-bromo-1(2H)-naphthalenone (Compound B) and 510.0 mg (2.90 mmol) of ethyl 4-vinylbenzoate in 4.0 ml of triethylamine (degassed by sparging with argon for 25 minutes), was added 124.0 mg (0.40 mmol) of tris(2-methylphenyl) phosphine, followed by 44.0 mg (0.20 mmol) of palladium(II)acetate. The resulting solution was heated to 95° C. for 2.5 hours, cooled to room temperature, and concentrated under reduced pressure. Purification by column chromatography (10% EtOAc/hexanes) afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 8.19 (1H, d, J=2.0 Hz), 8.03 (2H, d, J=8.4 Hz), 7.69 (1H, dd, J=2.0, 8.2 Hz), 7.57 (2H, d, J=8.4 Hz), 7.45 (1H, d, J=8.2 Hz), 7.20 (2H, s), 4.39 (2H, q, J=7.1 Hz), 2.76 (2H, t, J=6.5 Hz), 2.04 (2H, t, J=6.5 Hz), 1.41(3H, t, J=7.1 Hz, and 6H, s).

Ethyl (E)-4-[2-(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethenyl]-benzoate (Compound P)

To a cold (−78° C.) solution of 440.0 mg (2.40 mmol) of sodium bis(trimethylsilyl)amide in 10.0 ml of THF was added 700.0 mg (2.00 mmol) of ethyl (E)-4-[2-(5,6,7,8-tetrahydro-5,5-dimethyl-8-oxo-2-naphthalenyl)ethenyl]-benzoate (Compound O) as a solution in 25.0 ml of THF. After stirring at −78° C. for 1.5 hours, 960.0 mg (2.40 mmol) of 2[N,N-bis(trifluoromethylsulfonyl)amino]-5-chloropyridine was added in one portion. After 30 minutes the solution was warmed to 0° C. and stirred for 3 hours. The reaction was quenched by the addition of saturated aqueous NH_4Cl, and extracted with EtOAc. The combined extracts were washed with 5% aqueous NaOH, dried (Na_2SO_4), and the solvents removed under reduced pressure. The title compound was isolated as a colorless solid by column chromatography (7% EtOAc/hexanes). 1H NMR ($CDCl_3$): δ 8.04 (1H, d, J=8.4 Hz), 7.57 (2H, d, J=8.4 Hz), 7.52 (1H, s), 7.49 (1H, d, J=8.0 Hz), 7.33 (1H, d, J=8.0 Hz), 7.20 (1H, d, J=16.4 Hz), 7.10 (1H, d, J=16.4 Hz), 6.00 (1H, t, J=4.9 Hz), 4.39 (2H, q, J=7.1 Hz), 2.43 (2H, d, J=4.9 Hz), 1.41 (3H, t, J=7.1 Hz), 1.32 (6H, s).

Ethyl(E)-4-[2-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethenyl]-benzoate (Compound 44)

A solution of 4-lithiotoluene was prepared at −78° C. by the addition of 130.7 mg of t-butyllithium (2.04 mmol; 1.20 ml of a 1.7M solution in pentane) to a solution of 374.5 mg (2.20 mmol) of 4-bromotoluene in 2.5 ml of THF. After 30 minutes a solution of 313.4 mg (2.30 mmol) of $ZnCl_2$ in 2.0 ml of THF was added. The resulting solution was warmed to room temperature, stirred for 1.25 hour and then added via canula to a solution of 285.0 mg (0.590 mmol) of ethyl (E)-4-[2-(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethenyl]-benzoate (Compound P) and 29.0 mg (0.025 mmol) of tetrakis(triphenylphosphine)palladium(0) in 2.0 ml of THF. The resulting solution was stirred at room temperature for 1 hour and then at 55° C. for 2 hours. Upon cooling to room temperature the reaction was quenched by the addition of

saturated aqueous NH_4Cl. The mixture was extracted with EtOAc, and the combined extracts were washed with 5% aqueous NaOH, saturated aqueous NaCl, and dried over Na_2SO_4 before being concentrated under reduced pressure. The title compound was isolated by column chromatography (10% EtOAC/hexanes) as a colorless solid. 1H NMR ($CDCl_3$): δ 7.96 (2H, d, J=8.1 Hz), 7.47 (2H, d, J=8.1 Hz), 7.43–7.16 (7H, m), 7.07 (1H, d, J=16.3 Hz), 6.93 (1H, d, J=16.3 Hz), 5.97 (1H, t, J=4.7 Hz), 4.39 (2H, q, J=7.0 Hz), 2.41 (3H, s), 2.33 (1H, d, J=4.7 Hz), 1.38 (3H, t, J=7.0 Hz), 1.33 (6H, s).

(E)-4-[2-(5,6-Dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethenyl]-benzoic acid Compound 45

To a solution of 65.0 mg (0.190 mmol) of ethyl (E)4-[2-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethenyl]-benzoate (Compound 44) in 4.0 ml of THF was added 30.0 mg of LiOH (0.909 mmol, 1.0 ml of a 1.1M solution) and 1.0 ml of MeOH. The solution was heated to 55° C. for 3 hours, cooled to room temperature, and concentrated under reduced pressure. The residue was dissolved in H_2O and extracted with hexanes. The aqueous layer was acidified to pH 1 with 10% HCl, and extracted with Et_2O. The combined organic layers were washed with saturated aqueous NaCl, diluted with EtOAc to give a clear solution, and dried over Na_2SO_4. The solvents were removed under reduced pressure to give the title compound as a colorless solid. 1H NMR (d6-DMSO): δ 7.86 (2H, d, J=8.4 Hz), 7.66 (2H, d, J=8.4 Hz), 7.58 (1H, dd, J=1.7, 8.1 Hz), 7.41 (1H, d, J=8.1 Hz), 7.28 (1H, d, J=16.5 Hz), 7.23 (4H, s), 7.08 (1H, d, J=1.7 Hz), 7.07 (1H, d, J=16.5 Hz), 5.97 (1H, t, J=4.6 Hz), 2.35 (3H, s), 2.31 (1H, d, J=4.6 Hz), 1.29 (6H, s).

Ethyl 4-[2-(1,1-dimethyl-3-(4-methylphenyl)-5-indenyl) ethynyl]benzoate (Compound 47)

A solution of 32.0 mg (0.187 mmol) of 4- bromotoluene in 1.0 ml THF was cooled to −78° C. and 24.0 mg of t-butyllithium (0.375 mmol, 0.22 ml of a 1.7 M solution in pentane) was slowly added. The yellow solution was stirred for 30 minutes at which time 29.8 mg (0.219 mmol) of $ZnCl_2$ was added as a solution in 1.0 ml THF. The resulting solution was warmed to room temperature and after 30 minutes added to a second flask containing 29.0 mg (0.062 mmol) of ethyl 4-[2-(1,1-dimethyl-3-(trifluoromethylsulfonyl)oxy-5-indenyl) ethynyl]benzoate (Compound FF) and 2.9 mg (0.003 mmol) of tetrakis (triphenylphosphine)palladium (0) in 1.0 ml THF. The resulting solution was warmed to 50° C. for 1 hour and then stirred at room temperature for 4 hours. The reaction was quenched by the addition of saturated aqueous NH_4Cl, and then extracted with Et_2O. The combined organic layers were washed with water, saturated aqueous NaCl, and dried over $MgSO_4$ before being concentrated under reduced pressure. The title compound was isolated as a colorless oil by column chromatography (10% Et_2O/hexanes). 1H NMR (300 MHz, $CDCl_3$): δ 8.03 (2H, d, J=8.5 Hz), 7.66 (1H, s), 7.58 (2H, d, J=8.5 Hz), 7.50 (2H, d, J=8.0 Hz), 7.46 (1H, d, J=7.9 Hz), 7.38 (1H, d, J=7.7 Hz), 7.28 (2H, d, J=9 Hz), 6.43 (1H, s), 4.40 (2H, q, J=7.2 Hz), 2.43 (3H, s), 1.41 (3H, t;+6H, s).

4-[2-(1,1-dimethyl-3-(4-methylphenyl)-5- indenyl)ethynyl] benzoic acid(Compound 48)

To a solution of 10.0 mg (0.025 mmol) of ethyl 4-[2-(1, 1-dimethyl-3-(4-methylphenyl)-5- indenyl)ethynyl] benzoate (Compound 47) in 0.5 ml THF/H_2O (3:1 v/v) was added 5.2 mg (0.12 mmol) LiOH H_2O. After stirring at room temperature for 48 hours the solution was extracted with hexanes and the aqueous layer was acidified with saturated aqueous NH_4Cl. Solid NaCl was added and the resulting mixture extracted with EtOAc. The combined organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to give the title compound as a colorless solid. 1H NMR (300 MHz, d_6-DMSO): δ 7.95 (2H, d, J=8.3 Hz), 7.65 (2H, d, J=8.3 Hz), 7.57 (2H, m), 7.49 (3H, m), 7.30 (2H, d, J=7.9 Hz), 6.61 (1H, s), 2.36 (3H, s), 1.36 (6H, s).

3-(4-bromothiophenoxy)propionic acid

To a solution of 1.44 g (35.7 mmol) of NaOH in 20.0 ml degassed H_2O (sparged with argon) was added 6.79 g (35.7 mmol) of 4-bromothiophenol. The resulting mixture was stirred at room temperature for 30 minutes. A second flask was charged with 2.26 g (16.3 mmol) of K_2CO_3 and 15 ml of degassed H_2O. To this solution was added (in portions) 5.00 g (32.7 mmol) of 3- bromopropionic acid. The resulting potassium carboxylate solution was added to the sodium thiolate solution, and the resulting mixture stirred at room temperature for 48 hours. The mixture was filtered and the filtrate extracted with benzene, and the combined organic layers were dicarded. The aqueous layer was acidified with 10% HCl and extracted with EtOAc. The combined organic layers were washed with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting solid was recrystallized from Et_2O-hexanes to give the title compound as off-white crystals. 1H NMR ($CDCl_3$): δ 7.43 (2H, d, J=8.4 Hz), 7.25 (2H, d, J=8.4 Hz), 3.15 (2H, t, J=7.3 Hz), 2.68 (2H, t, J=7.3 Hz).

2,3-dihydro-6-bromo-(4H)-1-benzothiopyran-4-one

A solution of 3.63 g (13.9 mmol) of 3-(4-bromothiophenoxy)propionic acid in 60 ml methanesulfonic acid was heated to 75° C. for 1.5 hours. After cooling to room temperature the solution was diluted with H_2O and extracted with EtOAc. The combined organic layers were washed with 2N aqueous NaOH, H_2O, and saturated aqueous NaCl and then dried over $MgSO_4$. Removal of the solvent under reduced pressure afforded a yellow solid from which the product was isolated by column chromatography (3% EtOAc- hexanes) as a pale-yellow solid. 1H NMR ($CDCl_3$): δ 8.22 (1H, d, J=2.1 Hz), 7.48 1H, dd, J=2.1,8.3 Hz), 7.17 (1H, d, J=8.5 Hz), 3.24 (2H, t, J=6.4 Hz), 2.98 (2H, t, J=6.7 Hz).

2,3-dihydro-6-(2-trimethylsilylethynyl)-(4H)-1-benzothiopyran-4-one

A solution of 1.00 g (4.11 mmol) 2,3-dihydro-6- bromo-(4H)-1-benzothiopyran-4-one and 78.3 mg (0.41 mmol) CuI in 15.0 ml THF and 6.0 ml Et_2NH was sparged with argon for 5 minutes. To this solution was added 2.0 ml (1.39 g, 14.2 mmol) of (trimethylsilyl)acetylene followed by 288.5 mg (0.41 mmol) of bis(triphenylphosphine)palladium(II) chloride. The resulting dark solution was stirred at room temperature for 3 days and then filtered through a pad of Celite, which was washed with EtOAc. The filtrate was washed with H_2O and saturated aqueous NaCl before being dried over $MgSO_4$. The title compound was isolated as an orange oil by column chromatography (4% EtOA-hexanes). 1H NMR ($CDCl_3$): δ 8.13 (1H, d,J=1.9 Hz), 7.36 (1H, dd, J=2.1, 8.2 Hz), 7.14 (1H, d, J=8.2Hz), 3.19 (2H, d, J=6.3 Hz), 2.91 (2H, d, J=6.3 Hz), 0.21 (9H, s).

2,3-dihydro-6-ethynyl-(4H)-6-benzothiopyran-4-one

A solution containing 600.0 mg (2.25 mmol) of 2,3-dihydro-6-(2-trimethylsilylethynyl)-(4H)-1-benzothiopyran4-one and 100.0 mg (0.72 mmol) K_2CO_3 in 15 ml MeOH was stirred at room temperature for 20 hours. The solution was diluted with H_2O and extracted with Et_2O. The combined organic layers were washed with H_2O and saturated aqueous NaCl before being dried over $MgSO_4$. Removal of the solvents under reduced preesure afforded the title compound as an orange solid. 1H NMR ($CDCl_3$): δ 8.17

(1H, d, J=1.8 Hz), 7.40 (1H, dd, J=1.8, 8.2 Hz), 7.19 (1H, d, J=8.2 Hz), 3.22 (2H, t, J=6.3 Hz), 3.08 (1H, s) 2.94 (2H, t, J=6.3 Hz).

Ethyl 4-[2-(6-(2,3-dihydro-(4H)-1-benzothiopyran-4-onyl)) ethynyl]benzoate

A solution of 405.0 mg (2.15 mmol) 2,3-dihydro-6-ethynyl-(4H)-1-benzothiopyran-4-one and 594.0 mg (2.15 mmol) of ethyl 4-iodobenzoate in 15 ml Et_3N and 3 ml THF was sparged with argon for 15 minutes. To this solution was added 503.0 mg (0.72 mmol) of bis(triphenylphosphine) palladium(II) chloride and 137.0 mg (0.72 mmol) CuI. This solution was stirred for 20 hours at room temperature and then filtered through a pad of Celite, which was washed with EtOAc. Removal of the solvents under reduced pressure afforded a brown solid. Column chromatography (3% EtOA-hexanes) afforded the title compound as an orange solid. 1H NMR (d_6-acetone): δ 8.15 (1H, d, J=2.0 Hz), 8.02 (2H, d, J=8.5 Hz), 7.69 (2H, d, J=8.5 Hz), 7.61 (1H, dd, J=2.1, 8.3 Hz), 7.40 (1H, d, J=8.2 Hz), 4.35 (2H, q, J=7.1 Hz), 3.40 (2H, t, J=6.3 Hz), 2.96 (2H, t, J=6.3 Hz), 1.37 (3H, t, J=7.1 Hz).

Ethyl 4-[2-(6-(4-(trifluoromethylsulfonyl)oxy-(2H)-1-benzothiopyranyl))ethynyl]benzoate

To a solution of 221.9 mg (1.21 mmol) of sodium bis (trimethylsilyl)amide in 3.0 ml THF cooled to −78° C. was added 370.0 mg (1.10 mmol) of ethyl 4-[2-(6-(2,3-dihydro-(4H)-1-benzothiopyran-4-onyl))ethynyl]benzoate in 4.0 ml THF. After 30 minutes, a solution of 2-[N,N- bis (trifluoromethylsulfonyl)amino]-5-chloropyridine in 4.0 ml THF was slowly added. The reaction was slowly warmed to room temperature and after 5 hours quenched by the addition of saturated aqueous NH_4Cl. The mixture was extracted with EtOAc, and the combined organic layers were washed with 5% aqueous NaOH, H_2O, and saturated aqueous NaCl before being dried over MgSO4. Removal of the solvents under reduced pressure, followed by column chromatography (4% EtOAc-hexanes) afforded the title compound as a pale-yellow solid. 1H NMR (d_6-acetone): δ 8.12 (2H, d, J=8.5 Hz), 7.66 (2H, d, J=8.5 Hz), 7.56 (1H, d, J=1.7 Hz), 7.49 (1H, dd, J=1.7, 8.1 Hz), 7.40 (1H, d, J=8.1 Hz), 6.33 (1H, t, J=5.7 Hz), 4.35 (2H, q, J=7.1 Hz), 3.82 (2H, d, J=5.7 Hz), 1.37 (3H, t, J=7.1 Hz).

Ethyl 4-[2-(6-(4-(4-methylphenyl)-(2H)-1-benzothiopyranyl))ethynyl]benzoate (Compound 49)

To a solution of 120.8 mg (0.70 mmol) of 4- bromotoluene in 2.0 ml THF at −78° C. was added 88.4 mg (1.38 mmol, 0.81 ml of a 1.7 M solution in pentane) of t-butyllithium. After 30 minutes a solution of 131.6 mg (0.97 mmol) $ZnCl_2$ in 2.0 ml THF was added and the reulting pale-yellow solution warmed to room temperature. Stirring for 40 minutes was followed by addition of this solution to a second flask containing 129.2 mg (0.28 mmol) of ethyl 4-[2-(6-(4- (trifluoromethylsulfonyl)oxy-(2H)-1-benzothiopyranyl))ethynyl]benzoate, 14.0 mg (0.012 mmol) tetrakis(triphenylphosphine)palladium (0), and 2.0 ml THF. The resulting solution was heated to 50° C. for 5 hours, cooled to room temperature, and quenched by the addition of saturated aqueous NH_4Cl. The mixture was extracted with EtOAc, and the combined organic layers were washed with H_2O and saturated aqueous NaCl, then dried ($MgSO_4$) and concentrated to an orange oil. The title compound was isolated as a colorless solid by column chromatography (3 to 5% EtOAc-hexanes). 1H NMR (d_6-acetone): δ 7.98 (2H, d, J=8.3 Hz), 7.58 (2H, d, J=8.2 Hz), 7.44–7.38 (2H, m), 7.26–7.15 (5H, m), 6.14 (1H, t, J=5.8 Hz), 4.34 (2H, q, J=7.1 Hz), 3.53 (2H, d, J=5.8 Hz), 2.37 (2H, s), 1.35 (3H, t, J=7.1 Hz).

4-[2-(6-(4-(4-methylphenyl)-(2H)-1-benzothiopyranyl)) ethynyl]-benzoic acid (Compound 50)

To a solution of 29.0 mg (0.07 mmol) ethyl 4-[2-(6-(4-(4-methylphenyl)-(2H)-1 - benzothiopyranyl))ethynyl]benzoate (Compound 49) in 2.0 ml THF and 2.0 ml EtOH was added 160.0 mg (4.00 mmol, 2.0 ml of a 2 M aqueous solution). The resulting solution was stirred at 35° C. for 2 hours, and then cooled to room temperature and stirred an additional 2 hours. The reaction was quenched by the addition of 10% aqueous HCl and extracted with EtOAc. The combined organic layers were washed with H_2O and saturated aqueous NaCl, and dried over Na_2SO_4. Removal of the solvents under reduced pressure afforded a solid which was washed with CH_3CN and dried under high vacuum to give the title compound as a pale-yellow solid. 1H NMR (d_6-DMSO): δ 7.90 (2H, d, J=8.4 Hz), 7.59 (2H, d, J=8.4 Hz), 7.40 (4H, m), 7.25–7.13 (4H, m), 7.02 (1H, d, J=1.7 Hz), 6.11 (1H, t, J=5.7 Hz), 3.54 (2H, d, J=5.7 Hz), 2.34 (3H, s).

3,4-Dihydro-4,4-dimethyl-7-acetyl-1(2H)-naphthalenone (Compound R); and 3,4-dihydro-4,4-dimethyl-6-acetyl-1(2H)-nanhthalenone (Compound S)

To a cold (0° C.) mixture of aluminum chloride (26.3 g, 199.0 mmols) in dichloromethane (55 ml) was added acetylchloride (15 g, 192 mmols) and 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (24.4 g, 152 mmols) in dichloromethane (20 ml) over 20 minutes. The reaction mixture was warmed to ambient temparature and stirred for 4 hours. Ice (200 g) was added to the reaction flask and the mixture diluted with ether (400 ml). The layers were separated and the organic phase washed with 10% HCl (50 ml), water (50 ml), 10% aqueous sodium bicarbonate, and saturated aqueous NaCl (50 ml) before being dried over $MgSO_4$. Ths solvent was removed by distillation to afford a yellow oil which was dissolved in benzene (50 ml).

To a cold (0° C.) solution of acetic acid (240 ml) and acetic anhydride (120 ml) was added chromiumtrioxide (50 g, 503 mmols) in small portions over 20 minutes under argon. The mixture was stirred for 30 mins at 0° C. and diluted with benzene (120 ml). The benzene solution prepared above was added with stirring via an addition funnel over 20 minutes. After 8 hours, the reaction was quenched by careful addition of isopropanol (50 ml) at 0° C., followed by water (100 ml). After 15 minutes, the reaction mixture was diluted with ether (1100 ml) and water (200 ml), and then neutralized with solid sodium bicarbonate (200 g). The ether layer was washed with water (100 ml), and saturated aqueous NaCl (2x100 ml), and dried over $MgSO_4$. Removal of the solvent under reduced pressure afforded a mixture of the isomeric diketones which were separated by chromatography (5% EtOAc/hexanes). (Compound R): 1H NMR ($CDCl_3$): δ 8.55 (1H, d, J=2.0 Hz), 8.13 (1H, dd, J=2.0, 8.3 Hz), 7.53 (1H, d, J=8.3 Hz), 2.77 (2H, t, J=6.6 Hz), 2.62 (3H, s), 2.05 (2H, t, J=6.6 Hz), 1.41 (6H, s). (Compound S): 1H NMR ($CDCl_3$): δ 8.10 (1H, d, J=8.1 Hz), 8.02 (1H, d, J=1.6 Hz), 7.82 (1H, dd, J=1.6, 8.1 Hz), 2.77 (2H, t, J=7.1 Hz), 2.64 (3H, s), 2.05 (2H, t, J=7.1 Hz), 1.44 (6H, s). 3,4-Dihydro-4,4-dimethyl-7-(2-(2-methyl- 1,3-dioxolanyl))-1 (2H)-naphthalenone (Compound T)

A mixture of 3,4-dihydro-4,4-dimethyl-7-acetyl- 1(2H)-naphthalenone (Compound R) (140.0 mg, 0.60 mmol), ethylene glycol (55.0 mg, 0.90 mmol), p-toluenesulfonic acid monohydrate (4 mg) and benzene (25 ml) was refluxed using a Dean-Stark apparatus for 12 hours. The reaction was quenched by the addition of 10% aqueous sodium bicarbonate, and extracted with ether (2x75 ml). The combined organic layers were washed with water (5 ml), and

saturated aqueous NaCl (5 ml), and dried over $MgSO_4$. Removal of the solvent under reduced pressure afforded the title compound as an oil. 1H NMR ($CDCl_3$): δ 8.13 (1H, d, J=2.0 Hz), 7.64 (1H, dd, J=2.0, 8.2 Hz), 7.40 (1H, d, J=8.2 Hz), 3.97–4.10 (2H, m), 3.70–3.83 (2H, m), 2.73 (2H, t, J=6.5 Hz), 2.01 (2H, t, J=6.5 Hz), 1.64 (3H, s), 1.39 (6H, s).

1,2,3,4-Tetrahydro-1-hydroxy-1-(4-methylphenyl)-4,4-dimethyl-7-(2-(2-methyl-1,3-dioxolanyl))naphthalene (Compound U)

To a solution of 195.4 mg (1.00 mmol) p- tolulylmagnesiumbromide (1.0 ml; 1M solution in ether) in 2 ml THF was added a solution of 3,4-dihydro-4,4-dimethyl-7-(2-(2-methyl-1,3-dioxolanyl))-1(2H)- naphthalenone (Compound T) 135.0 mg, 0.52 mmol) in 5 ml THF. The solution was refluxed for 16 hours, cooled to room temperature, and diluted with ether (50 ml). The solution was washed with water (5 ml), saturated aqueous NH_4Cl (5 ml), and dried over $MgSO_4$. Removal of the solvents under reduced pressure and column chromatography (5% EtOAc/hexanes) afforded the title compound as a solid. 1H NMR ($CDCl_3$): δ 7.37 (2H, d), 7.21 (1H, s), 7.13 (2H, d, J=8.5 Hz), 7.08 (2H, d, J=8.5 Hz), 3.88–3.99 (2H, m), 3.58–3.75 (2H, m), 2,34 (3H, s), 2.12–2.30 (2H, m), 1.79–1.90 (1H, m), 1.57 (3H, s), 1.48–1.58 (1H, m), 1.38 (3H, s), 1.31 (3H, s).

3,4-Dihydro-1-(4-methylphenyl)-4,4-dimethyl-7-acetylnaphthalene (Compound V)

A mixture of 1,2,3,4-tetrahydro-1 -hydroxy-1-(4-methylphenyl)-4,4-dimethyl-7-(2-(2-methyl-1,3-dioxolanyl))naphthalene (Compound U) 130.0 mg (0.38 mmol), p-toluenesulfonic acid monohydrate (4 mg) and benzene (5 ml) was refluxed for 16 hours. Upon cooling to room temperature, the reaction mixture was diluted with ether (100 ml) and washed with 10% aqueous sodium bicarbonate, water, and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and the solvents were removed under reduced pressure to give the title compound as a solid. 1H NMR ($CDCl_3$): δ 7.83 (1H, dd, J=1.8,8.0 Hz), 7.66 (1H, d, J=1.8 Hz), 7.45 (1H, d, J=8.0 Hz), 7.25 (2H, d, J=8.5 Hz), 7.22 (2H, d, J=8.5 Hz), 6.03 (1H, t, J=6.3Hz), 2.47 (3H, s), 2.41 (3H, s), 2.37 (2H, d, J=6.3 Hz), 1.36 (6H, s).

(E)-3-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)-2-butenenitrile (Compound W)

To a slurry of NaH (48.0 mg, 2.00 mmol) in THF (6 ml), was added diethylcyanomethylphosphonate (450.0 mg, 2.50 mmol). After 40 mins, a solution of 3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-7-acetylnaphthalene (Compound V) 95.0 mg, (0.33 mmol) in THF (4 ml) was added. The mixture was stirred for 16 hours, diluted with ether (100 ml), and washed with water, and saturated aqueous NaCl before being dried over $MgSO_4$. Removal of the solvents under reduced pressure, and column chromatography (3% EtOAc/hexanes) afforded the title compound as a solid. 1H NMR ($CDCl_3$): δ 7.39 (1H, d, J=1H), 7.32 (1H, dd, J=2.0, 8.1Hz), 7.20–7.25 (4H, brs), 7.15 (1H, d, J=2.0 Hz), 6.03 (1H, t, J=6.0 Hz), 5.44 (1H, s), 2.42 (3H, s), 2.36 (2H, d, J=6.0 Hz), 2.35 (3H, s), 1.35 (6H, s).

(E)-3-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)-2-butenal (Compound X)

To a cold solution (−78° C.) of (E)-3-(5,6-dihydro- 5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)-2- butenenitrile (Compound W) 84.0 mg, 0.29 mmol) in dichloromethane (4 ml) was added 0.50 ml (0.50 mmol) of diisobutylaluminumhydride (1M solution in dichloromethane). After stirring for 1 hour, the reaction was quenched at −78° C. by adding 2-propanol (1 ml) diluted with ether (100 ml). Upon warming to room temperature, the solution was washed with water, 10% HCl, and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure to give the title compound as an oil. 1H NMR ($CDCl_3$): δ 10.12 (1H, d, J=7.9 Hz), 7.43 (2H, s), 7.19–7.28 (5H, m), 6.27 (1H, d, J=7.9 Hz), 6.03 (1H, t, J=4.8 Hz), 2.47 (3H, s), 2.42 (3H, s), 2.37 (2H, d, J=4.8 Hz), 1.37 (6H, s).

Ethyl (E,E,E)-3-methyl-7-(5,6-dihydro-5,5-dimethyl-8-(4-methylyhenyl)-2-naphthalenyl)-2,4,6-octatrienoate (Compound 51)

To a cold (−78° C.) solution of diethyl-(E)-3-ethoxycarbonyl-2-methylallylphosphonate [prepared in accordance with *J Org. Chem.* 39: 821 (1974)] 264.0 mg, (1.00 mmol) in THF (2 ml) was added 26.0 mg (0.41 mmol, 0.65 ml) of n-butyllithium in hexanes (1.6 M solution) followed immediately by the addition of (E)-3-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)-2-butenal (Compound X) 82.0 mg, 0.26 mmol) in THF (3 ml). After 1 hour, the reaction mixture was diluted with ether (60 ml), washed with water (5 ml), saturated aqueous NaCl (5 ml) and dried over $MgSO_4$. After removal of the solvents under reduced pressure, the title compound was isolated as an oil by column chromatography (5% EtOAc/hexanes, followed by HPLC using 1% EtOAc/hexanes). 1H NMR (acetone-d6): δ 7.36–7.43 (2H, m), 7.18–7.27 (4H, m), 7.17 (1H, d, J=1.7 Hz), 7.08 (1H, dd, J=11.2, 15.2 Hz), 6.46 (1H, d, J=11.2 Hz), 6.38 (1H, d, J=15.2 Hz), 5.98 (1H, t, J=4.7 Hz), 5.78 (1H, s), 4.10 (2H, q, J=7.1 Hz), 2.35 (3H, s), 2.33 (3H, s), 2.32 (2H, d, J=4.7 Hz), 2.12 (3H, s), 1.31 (6H, s), 1.22 (3H, t, J=7.1 Hz).

(E,E,E)-3-methyl-7-(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)-2,4,6-octatrienoic acid (Compound 52)

To a solution of ethyl (E,E,E)-3-methyl-7-(5,6- dihydro-5,5-dimethyl-8-(4-methylphenyl)-2- naphthalenyl)-2,4,6-octatrienoate (Compound 51) 85.0 mg, 0.20 mmol) in THF (1 ml) and methanol (1 ml) was added 12.0 mg (0.50 mmol) of LiOH (0.5 ml, 1M solution). The mixture was stirred for 6 hours, diluted with ether (60 ml), acidified with 10% HCl (1 ml). The solution was washed with water, and saturated aqueous NaCl, before being dried over $MgSO_4$. Removal of the solvents under reduced pressure afforded the title compound as a solid, which was purified by recrystallization from acetone. 1H NMR (acetone-d6): δ 7.35–7.45 (2H, m), 7.19–7.28 (4H, m), 7.17 (1H, d, J=1.8 Hz), 7.09 (1H, dd, J=11.5, 15.1 Hz), 6.48 (1H, d, J=11.5 Hz), 6.42 (1H, d, J=15.1 Hz),5.99 (1H, t, J=4.7 Hz),5.82 (1H, s), 2.36 (3H, s), 2.33 (2H, d, J=4.7 Hz), 2.32 (3H, s), 2.13 (3H, s), 1.32 (6H, s).

3,4-dihydro-4,4-dimethyl-7-nitro-1(2H)-naphthalenone (Compound Y)

To 1.7 ml (3.0 g, 30.6 mmol, 18M) H_2SO_4 at −5° C. (ice-NaCl bath) was slowly added 783.0 mg (4.49 mmol) of 3,4-dihydro-4,4-dimethyl-1(2H)-naphthalenone. A solution of 426.7 mg (6.88 mmol, 0.43 ml, 16M) HNO_3, and 1.31 g (0.013 mol, 0.74 ml, 18 M) H_2SO_4 was slowly added. After 20 minutes, ice was added and the resulting mixture extracted with EtOAc. The combined extracts were concentrated under reduced pressure to give a residue from which the title compound, a pale yellow solid, was isolated by column chromatography (10% EtOAC/hexanes). 1H NMR ($CDCl_3$): δ 8.83 (1H, d, J=2.6 Hz), 8.31 (1H, dd, J=2.8, 8.9 Hz), 7.62 (1H, d, J=8.7 Hz), 2.81 (2H, t, J=6.5 Hz), 2.08 (2H, t, J=6.5 Hz), 1.45 (6H, s).

3,4-dihydro-4,4-dimethyl-7-amino-1(2H)-naphthalenone (Compound Z)

A solution of 230.0 mg (1.05 mmol) 3,4-dihydro-4,4-dimethyl-7-nitro-1(2H)-naphthalenone (Compound Y) in

5.0 ml of EtOAc was stirred at room temperature with a catalytic amount of 10% Pd—C under 1 atm of H_2 for 24 hours. The catalyst was removed by filtration through a pad of Celite, and the filtrate concentrated under reduced pressure to give the title compound as a dark green oil. 1H NMR ($CDCl_3$): δ 7.30 (1H, d, J=2.7 Hz), 7.22 (1H, d, J=8.4 Hz), 6.88 (1H, dd, J=2.7, 8.5 Hz), 2.70 (2H, t, J=6.6 Hz), 1.97 (2H, t, J=6.6 HZ), 1.34 (6H, s).

Ethyl 4-[(5,6,7,8-tetrahydro-5,5-dimethyl-8-oxo-2-naphthalenyl)azo]-benzoate (Compound AA)

To a solution of 198.7 mg (1.05 mmol) 3,4-dihydro-4,4-dimethyl-7-amino-1(2H)-naphthalenone (Compound Z) in 5.0 ml glacial acetic acid was added 180.0 mg (1.00 mmol) of ethyl 4-nitrosobenzoate. The resulting solution was stirred overnight at room temperature, and then concentrated under reduced pressure. The product was isolated from the residual oil as a red solid, by column chromatography (15% EtOA-hexanes). 1H NMR ($CDCl_3$): δ 8.57 (1H, d, J=2.0 Hz), 8.19 (2H, d, J=8.4 Hz), 8.07 (1H, d, J=8.0 Hz), 7.94 (2H, d, J=8.4 Hz), 7.58 (1H, d, J=8.6 Hz), 4.41 (2H, q, J=7.1 Hz), 2.79 (2H, t, J=6.6 Hz), 2.07 (2H, t, J=7.02 Hz), 1.44 (6H, s), 1.42 (3H, t, J=7.1 Hz).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)azo]-benzoate (Compound BB)

To a solution of 90.4 mg sodium bis(trimethylsilyl)amide (0.48 mmol, 0.48 ml of a 1.0 M THF solution) in 2.0 ml THF at −78° C., was added 153.0 mg (0.437 mmol) of ethyl 4-[(5,6,7,8-tetrahydro-5,5- dimethyl-8-oxo-2-naphthalenyl) azo]-benzoate (Compound AA) in 2.0 ml THF. The dark red solution was stirred at −78° C. for 30 minutes and then 204.0 mg (0.520 mmol) of 2-[N,N-bis(trifluoromethylsulfonyl) amino]-5-chloropyridine was added as a solution in 2.0 ml THF. The reaction mixture was allowed to warm to room temperature and after 3 hours it was quenched by the addition of H_2O. The organic layer was concentrated to a red oil under reduced pressure. The product was isolated by column chromatography (25% EtOAc/hexanes) as a red oil. 1H NMR ($CDCl_3$): δ 8.21 (2H, d, J=8.6 Hz), 7.96 (2H, d, J=8.6 Hz), 7.94 (2H, m), 7.49 (1H, d, J=8.2 Hz), 6.08 (1H, t, J=2.5 Hz), 4.42 (2H, q, J=7.1 Hz), 2.49 (2H, d, J=4.8 Hz), 1.44 (3H, t, J=7.1 Hz), 1.38 (6H, s).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl- 8-(4-methylphenyl)-2-naphthalenyl)azo]-benzoate (Compound 46a)

A solution of 4-lithiotoluene was prepared by the addition of 62.9 mg (0.58 ml, 0.98 mmol) of t-butyl lithium (1.7 M solution in pentane) to a cold solution (−78° C.) of 84.0 mg (0.491 mmol) of 4-bromotoluene in 1.0 ml of THF. After stirring for 30 minutes a solution of 107.0 mg (0.785 mmol) of zinc chloride in 2.0 ml of THF was added. The resulting solution was warmed to room temperature, stirred for 30 minutes, and added via cannula to a solution of 94.7 mg (0.196 mmol) of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)azo]-benzoate (Compound BB) and 25 mg (0.02 mmol) of tetrakis (triphenylphosphine)palladium(0) in 2.0 ml of THF. The resulting solution was heated at 50° C. for 1.5 hours, cooled to room temperature and diluted with sat. aqueous NH_4Cl. The mixture was extracted with EtOAc (40 ml) and the combined organic layers were washed with water and brine. The organic phase was dried over Na_2SO_4, concentrated in vacuo, and the title compound isolated as a red solid by column chromatography (25% EtOAc-hexanes) 1H NMR ($CDCl_3$): δ 8.21 (2H, d, J=8.6 Hz), 7.96 (2H, d, J=8.6 Hz), 7.94 (2H, m), 7.49 (1H, d, J=8.2 Hz), 6.08 (1H, t, J=2.5 Hz), 4,42 (2H, q, J=7.1 Hz), 2.49 (2H, d, J=4.8 Hz), 1.44 (3H, t, J=7.1 Hz), 1.38 (6H, s).

4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)azo]-benzoic acid (Compound 46b)

To a solution of ethyl 4-[(5,6-dihydro-5,5- dimethyl-8-(4-methylphenyl)-2-naphthalenyl)azo]- benzoate (Compound 46a) 16.5 mg, 0.042 mmol) in THF (2 ml) and ethanol (1ml) was added 80.0 mg (2.00 mmol) of NaOH (2.0 ml, 1M aqueous solution). The mixture was stirred for 12 hours at room temperature, acidified with 10% HCl, and extracted with EtOAc. The combined organic layers were washed with water, and saturated aqueous NaCl, then dried over $MgSO_4$. Removal of the solvents under reduced pressure, and recrystallization of the residue from EtOAC/hexane, afforded the title compound as a red solid. 1H NMR (acetone-d6): δ 8.19 (2H, d, J=8.4 Hz), 7.92 (2H, d, J=8.5 Hz), 7.88 (2H, dd, J=2.1, 6.1 Hz), 7.66 (1H, s), 7.64 (2H, d, J=2.3 Hz), 7.28 (4H, d, J=3.0 Hz), 6.09 (1H, t, J=2.5 Hz), 2.42 (2H, d, J=4.8 Hz), 2.39 (3H, s), 1.40 (6H, s).

6-(2-Trimethylsilyl)ethynyl-2,3-dihydro-3,3 -dimethyl-1H-inden-1-one (Compound CC)

To a solution of 815.0 mg (3.41 mmol) 6-bromo-2,3-dihydro-3,3-dimethyl-1H-inden-1-one (See Smith et al. Org. Prep. Proced. Int. 1978 10 123–131) in 100 ml of degassed Et_3N (sparged with argon for 20 min) was added 259.6 mg (1.363 mmol) of copper(I) iodide, 956.9 mg (1.363 mmol) of bis(triphenylphosphine)palladium(II)chloride, and 3.14 g (34.08 mmol) of (trimethylsilyl)acetylene. This mixture was heated at 70° C. for 42 hours, cooled to room temperature, and filtered through a pad of silica gel and washed with ether. The filtrate was washed with water, 1 M HCl, water, and finaly with saturated aqueous NaCl before being dried over $MgSO_4$. Concentration of the solution under reduced pressure, followed by column chromatography (silica gel; 10% Et_2O-hexanes) afforded the title compound as a brown oil. 1H NMR (300 MHz, $CDCl_3$): δ 7.79(1H, d, J=1.4 Hz), 7.69 (1H, dd, J=1.6, 8.3 Hz), 7.42 (1H, d, J=8.5 Hz), 2.60 (2H, s), 1.41 (6H, s), 0.26 (9H, s).

6-Ethynyl-2,3-dihydro-3,3-dimethyl-1H-inden-1-one (Compound DD)

To a solution of 875.0 mg (3.41 mmol) 6-(2-trimethylsilyl)ethynyl-2,3-dihydro-3,3-dimethyl-1H-inden-1-one (Compound CC) in 28 ml of MeOH, was added 197.3 mg (1.43 mmol) of K_2CO_3 in one portion. After stirring for 6 hours at room temperature the mixture was filtered though a pad of Celite and the filtrate concentrated under reduced pressure. The residual oil was placed on a silica gel column and eluted with 5% EtOAc-hexanes to give the title product as a colorless oil. 1H NMR (300 MHz, CDCl13): δ 7.82 (1H, s), 7.72 (1H, dd, J=1.6, 7.8 Hz), 7.47 (1H, d, J=8.4 Hz), 3.11 (1H, s), 2.61 (2H, s), 1.43 (6H, s).

Ethyl 4-[2-(5,6-dihydro-5,5-dimethyl-7-oxo-2-indenyl) ethynyl]benzoate (Compound EE)

A solution of 280.0 mg (1.520 mmol) 6-ethynyl-2,3-dihydro-3,3-dimethyl-1H-inden-1-one (Compound DD) and 419.6 mg (1.520 mmol) ethyl 4-iodobenzoate in 5 ml Et_3N was sparged with argon for 40 minutes. To this solution was added 271.0 mg (1.033 mmol) of triphenylphosphine, 53.5 mg (0.281 mmol) of copper(I) iodide, and 53.5 mg (0.076 mmol) of bis(triphenylphosphine)palladium(II) chloride. The resulting mixture was heated to reflux for 2.5 hours, cooled to room temperature, and diluted with Et_2O. After filtration through a pad of Celite, the filtrate was washed with H_2O, M HCl, H_2O, and saturated aqueous NaCl, then dried over $MgSO_4$, and concentrated under reduced pressure. The title compound was isolated as a pale-yellow solid by column chromatography (15% EtOAc-hexanes). 1H NMR (300 MHz, d6-acetone): δ 8.05 (2H, d, J=8.6 Hz), 7.87 (1H, dd, J=1.4, 8.1 Hz), 7.75 (2H, m), 7.70 (2H, d, J=8.5

Hz), 4.36 (2H, q, J=7.1 Hz), 2.60 (2H, s), 1.45 (6H, s), 1.37 (3H, t, J=7.1 Hz).

Ethyl 4-[2-(1,1-dimethyl-3-(trifluoromethyl-sulfonyl)oxy-5-indenyl)ethynyl]benzoate (Compound FF)

A solution of 88.0 mg (0.48 mmol) of sodium bis (trimethylsilyl)amide in 0.5 ml THF was cooled to -78° C. and 145.0 mg (0.436 mmol) of ethyl 4-[2-(5,6-dihydro-5,5-dimethyl-7-oxo-2-indenyl)ethynyl]benzoate (Compound EE) was added as a solution in 1.0 ml THF. After 30 minutes 181.7 mg (0.480 mmol) of 2-(N,N-bis (trifluoromethansulfonyl)amino)-5-chloro-pyridine was added as a solution in 1.0 ml THF. The reaction was allowed to slowly warm to room temperature and quenched after 5 hours by the addition of saturated aqueous NH_4Cl. The mixture was extracted with EtOAc, and the combined organic layers washed with 5% aqueous NaOH, H_2O, and saturated aqueous NaCl, then dried ($MgSO_4$) and concentrated under reduced pressure. The product was isolated as a colorless solid by column chromatography (10% Et_2O-hexanes). 1H NMR (300 MHz, d6-acetone): δ 8.05 (2H, d, J=8.3 Hz), 7.69 (2H, d, J=8.4 Hz), 7.63 (2H, s), 7.55 (1H, s), 4.36 (2H, q, J=7.1 Hz), 1.44 (6H, s), 1.37 (3H, t, J=7.1 Hz).

4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 60)

A solution of 142.6 mg (0.339 mmol) of ethyl 4- [(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2- naphthalenyl) ethynyl]benzoate (Compound 1) and 35.6 mg (0.848 mmol) of LiOH—H_2O in 12 ml of THF/water (4:1, v/v), was stirred overnight at room temperature. The reaction mixture was extracted with hexanes, and the hexane fraction extracted with 5% aqueous NaOH. The aqueous layers were combined and acidified with 1M HCl, and then extracted with EtOAc and Et_2O. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give the title compound as a colorless solid. 1H NMR (d_6-DMSO): δ 7.91 (2H, d, J=8.4 Hz), 7.60 (2H, d, J=8.4 Hz), 7.47 (2H, s), 7.23 (4H, q, J=8.1 Hz), 7.01 (1H, s), 6.01 (1H, t, J=4.6 Hz), 2.35 (3H, s), 2.33 (2H, d, J=4.8 Hz), 1.30 (6H, s).

4-[(5,6-dihydro-5,5-dimethyl-8-phenyl-2-naphthalenyl) ethynyl]benzoic acid (Compound 60a)

Employing the same general procedure as for the preparation of 4-[(5,6-dihydro-5,5-dimethyl-8-(2- thiazolyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 30a), 27.0 mg (0.07 mmol) of ethyl 4-[(5,6- dihydro-5,5-dimethyl-8-phenyl-2-naphthalenyl)ethynyl]benzoate (Compound 1a) was converted into the title compound (colorless solid) using 5.9 mg (0.14 mmol) of LiOH in H_2O. PMR (d6-DMSO): δ 1.31 (6H, s), 2.35 (2H, d, J=4.5 Hz), 6.05 (1H, t, J=J=J=4.5 Hz), 7.00 (1H, s), 7.33 (2H, d, J=6.2 Hz), 7.44 (4H, m), 7.59 (2H, d, J=8.1 Hz), 7.90 (2H, d, J=8.1 Hz).

4-[(5,6-Dihydro-5,5-dimethyl-8-(4-(1,1-dimethylethyl) phenyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 61)

A solution of 80.0 mg (0.173 mmol) of ethyl 4- [(5,6-dihydro-5,5-dimethyl-8-(4-(1,1- dimethylethyl)phenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 6) and 18.1 mg (0.432 mmol) of LiOH—H_2O in 6 ml of THF/water (3:1, v/v), was stirred overnight at room temperature. The reaction mixture was extracted with hexanes, and the remaining aqueous layer acidified with 1M HCl, and then extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to give the title compound as a colorless solid. 1H NMR (d_6-DMSO): δ 7.82 (2H, d, J=8.2 Hz), 7.44 (6H, m), 7.25 (2H, d, J=8.3 Hz), 7.02 (1H, s), 6.01 (1H, t, J=4.6 Hz), 2.32 (2H, d, J=4.7 Hz), 1.32 (9H, s), 1.29 (6H, s).

Ethyl 2-fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)thiocarbonyl]amino]-benzoate (Compound 62)

A solution of 54.4 mg (0.119 mmol) ethyl 2-fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)carbonyl]amino]-benzoate (Compound 40) and 57.7 mg (0.143 mmol) of [2,4-bis(4- methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4- disulfide] (Lawesson's Reagent) in 12.0 ml of benzene was refluxed overnight. Upon cooling to room temperature, the mixture was filtered and the filtrate concentrated under reduced pressure. The title compound was isolated by column chromatography (10 to 25% EtOAc/hexanes) as a yellow solid. 1H NMR ($CDCl_3$): δ 9.08 (1H, s), 7.92 (1H, br s), 7.90 (1H, t, J=8.2 Hz), 7.66 (1H, dd, J=2.0, 6.0 Hz), 7.38 (3H, m), 7.18 (4H, m), 6.01 (1H, t, J=4.7 Hz), 4.35 (2H, q, J=7.1 Hz), 2.36 (3H, s), 2.33 (2H, d, J=4.7 Hz), 1.38 (3H, t, J=7.1 Hz), 1.33 (6H, s).

2-fluoro-4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)thiocarbonyl]amino]-benzoic acid (Compound 63)

To a solution of 46.5 mg (0.098 mmol) ethyl 2- fluoro4-[[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)thiocarbonyl]amino]-benzoate (Compound 62) in 1.0 ml EtOH and 1.0 ml of THF was added 55 mg NaOH (1.4 mmol) and 1.0 ml of H_2O. After stirring at room temperature for overnight EtOAc was added, and the reaction quenched by the addition of 10% HCl. Extraction with EtOAc was followed by washing of the combined organic layers with H_2O, saturated aqueous NaCl, and drying over $MgSO_4$. Removal of the solvent under reduced pressure provided a solid which after crystallization from CH_3CN afforded the title compound as a pale-yellow solid. 1H NMR (d_6-acetone): δ 11.05 (1H, s), 8.02 (1H, m), 7.99 (1H, t, J=8.3 Hz), 7.75 (1H, m), 7.69 (1H, dd, J=2.0, 6.1 Hz), 7.52 (1H, s), 7.46 (1H, d, J=8.1 Hz), 7.21 (4H, m), 6.04 (1H, t, J=4.8 Hz), 2.37 (2H, d, J=4.8 Hz), 2.33 (3H, s), 1.36 (6H, s).

Ethyl 5',6'-dihydro-5',5'-dimethyl-8'-(4-methylphenyl)-[2,2'-binaphthalene]-6-carboxylate (Compound 64)

A solution of 3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-7-bromonaphthalene Compound D) 0.45 g, 1.40 mmol) and THF (2.1 ml) was added to magnesium turnings (0.044 g, 1.82 mmol) at room temperature under argon. Two drops of ethylene dibromide were added, and the solution, which slowly became cloudy and yellow, was heated to reflux for 1.5 hours. In a second flask was added zinc chloride (0.210 g, 1.54 mmol), which was melted under high vacuum, cooled to room temperature and dissolved in THF (3 ml). The Grignard reagent was added to the second flask and, after 30 minutes at room temperature, a solution of ethyl 6-bromo-2-naphthalinecarboxylate (Compound N) 0.293 g, (1.05 mmol) and THF (2 ml) were added. In a third flask was prepared a solution of $Ni(PPh_3)_4$ and THF as follows: To a solution of $NiCl_2(PPh_3)_2$ (0.82 g, 1.25 mmol) and PPh_3 (0.66 g, 2.5 mmol) in THF (3.5 ml) was added a 1M solution of diisobutylaluminum hydride and hexanes (2.5 ml, 2.5 mmol), and the resulting solution diluted with THF to a total volume of 15 ml and stirred at room temperature for 15 minutes. Three 0.60 ml aliquots of the $Ni(PPh_3)_4$ solution were added at 15 minutes intervals to the second flask. The resulting suspension was stirred at room temperature for 2 hours. The reaction was quenched by the addition of 5 ml 1N aqueous HCl and stirred for 1 hour before extracting the products with ethyl acetate. The organic layers were combined, washed with brine, dried ($MgSO_4$), filtered and the solvent removed in-vacuo. The residue was crystalized from hexanes to give 130 mg of pure

material. The mother liquor was concentrated under reduced pressure and the residue purified by silica gel chromatography (95:5- hexanes:ethyl acetate) to give an additional 170 mg of the title compound (overall yield=300 mg, 64 %) as a colorless solid. 1H NMR ($CDCl_3$) δ8.57 (s, 1H), 8.05 (dd, 1H, J=1.7, 8.0 Hz), 7.84–7.95 (overlapping d's, 3H), 7.66 (dd, 1H, J=1.7, 8.5 Hz), 7.58 (dd, 1H, J=2.0, 8.0 Hz), 7.48 (d, 1H, J=8.0 Hz), 7.43 (d, 1H, J=2.0 Hz), 7.32 (d, 2H, J=8.0 Hz), 7.21 (d, 2H, J=8.0 Hz), 6.04 (t, 1H, J=4.8 Hz), 4.44 (q, 2H, J=7.1 Hz), 2.40 (s, 3H), 2.39 (d, 2H, J=4.8 Hz), 1.45 (t, 3H, J=7.1 Hz), 1.39 (s, 6H).

5',6'-Dihydro-5',5'-dimethyl-8'-(4-methylphenyl)-[2,2'-binaphthalene]-6-carboxylic acid (Compound 65)

A solution of ethyl 5',6'-dihydro-5',5'-dimethyl- 8'-(4-methylphenyl)-[2,2'-binaphthalene]-6-carboxylate (Compound 64) 0.19 g, 0.43 mmol), EtOH (8 ml) and 1N aqueous NaOH (2 ml) was heated to 60° C. for 3 hours. The solution was cooled to 0° C. and acidified with 1N aqueous HCl. The product was extracted into ethyl acetate, and the organic layers combined, washed with water, brine, dried ($MgSO_4$), filtered and the solvent removed in-vacuo. The residue was recrystalized from THF/ethyl acetate at 0° C. to give 35 mg of pure material. The mother liquor was concentrated under reduced pressure and the residue purified by silica gel chromatography (100% ethyl acetate) to give an additional 125 mg of the title compound (overall yield=160 mg, 90%) as a colorless solid. 1H NMR (DMSO-d_6) δ8.57 (s, 1H), 8.11 (d, 1H, J=8.7 Hz), 7.96–7.82 (overlapping d's, 3H),7.65 (d, 2H, J=7.6 Hz), 7.50 (d, 1H, J=7.9 Hz), 7.28 (s, 1H), 7.26 (d, 2H, J=8.3 Hz), 7.21 (d, 2H, J=8.3 Hz), 6.01 (t, 1H, J=4.5 Hz), 3.34 (br s, 1H), 2.31 (s, 3H), 2.31 (d, 2H, J=4.5 Hz), 1.31 (s, 6H).

Ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(2-furyl)-2-naphthalenyl)ethynyl]benzoate (Compound 66)

Employing the same general procedure as for the preparation of ethyl 4-[(5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl)ethynyl]benzoate (Compound 1), 250.0 mg (0.52 mmol) of ethyl 4-[(5,6-dihydro-5,5- dimethyl-8-(trifluoromethylsulfonyl)oxy-2-naphthalenyl)ethynyl]benzoate (Compound G) was converted into the title compound (colorless solid) using 142.4 mg (1.045 mmol) of zinc chloride, 24.1 mg (0.02 mmol) of tetrakis(triphenylphosphine)palladium(0) and 2-lithiofuran (prepared by the addition of 53.4 mg (0.52 ml, 0.78 mmol) of n-butyllithium (1.5M solution in hexane) to a cold solution (–78° C.) of 53.4 mg (0.784 mmol) of furan in 1.0 ml of THF). PMR ($CDCl_3$): δ 1.32 (6H, s), 1.41 (3H, t, J=7.1 Hz), 2.35 (2H, d, J=5.0 Hz), 4.39 (2H, q, J=7.1 Hz), 6.41 (1H, t, J=5.0 Hz), 6.50 (2H, s), 7.36 (1H, d, J=8.0 Hz), 7.45 (1H, dd, J=1.7, 8.0 Hz), 7.49 (1H, s), 7.57 (2H, d, J=8.2 Hz), 7.63 (1H, d, J=1.7 Hz), 8.02 (2H, d, J=8.2 Hz).

4-[(5,6-dihydro-5,5-dimethyl-8-(2-furyl)-2-naphthalenyl) ethynyl]benzoic acid (Compound 67)

Employing the same general procedure as for the preparation of 4-[(5,6-dihydro-5,5-dimethyl-8-(2- thiazolyl)-2-naphthalenyl)ethynyl]benzoic acid (Compound 30a), ethyl 4-[(5,6-dihydro-5,5-dimethyl-8- (2-furyl)-2-naphthalenyl) ethynyl]benzoate (Compound 66) was converted into the title compound (colorless solid) using 16.0 mg (0.38 mmol) of LiOH in H_2O. PMR (d_6-DMSO): δ 1.26 (6H, s), 2.33 (2H, d, J=4.9 Hz), 6.41 (1H, t, J=4.9 Hz), 6.60 (2H, m), 7.45–7.53 (3H, m), 7.64 (2H, d, J=8.3 Hz), 7.75 (1H, d, J=1.6 Hz), 7.93 (2H, d, J=8.3 Hz).

3,4-dihydro-4,4-dimethyl-7-acetyl-1(2H)-naphthalenone (Compound 100C) and 3,4-dihydro-4,4-dimethyl-6-acetyl-1(2H)-naphthalenone (Compound 100D)

To a cold (0°C.) mixture of aluminum chloride (26.3 g, 199.0 mmols) in dichloromethane (55 ml) was added acetylchloride (15 g, 192 mmols) and 1,2,3,4-tetrahydro-1,1-dimethylnaphthalene (24.4 g, 152 mmols) in dichloromethane (20 ml) over 20 minutes. The reaction mixture was warmed to ambient temparature and stirred for 4 hours. Ice (200 g) was added to the reaction flask and the mixture diluted with ether (400 ml). The aqueous and organic layers were separated and the organic phase was washed with 10% HCl (50 ml), water (50 ml), 10% aqueous sodium bicarbonate, and saturated aqueous NaCl (50 ml) and then dried over $MgSO_4$. The solvent was removed by distillation to afford a yellow oil which was dissolved in benzene (50 ml).

To a cold (0° C.) solution of acetic acid (240 ml) and acetic anhydride (120 ml) was added chromium trioxide (50 g, 503 mmols) in small portions over 20 minutes under argon. The mixture was stirred for 30 minutes at 0° C. and diluted with benzene (120 ml). The benzene solution prepared above was added with stirring via an addition funnel over 20 minutes. After 8 hours, the reaction was quenched by the careful addition of isopropanol (50 ml) at 0° C., followed by water (100 ml). After 15 minutes, the reaction mixture was diluted with ether (1100 ml) and water (200 ml), and then neutralized with solid sodium bicarbonate (200 g). The ether layer was washed with water (100 ml), and saturated aqueous NaCl (2x100 ml), and dried over $MgSO_4$. Removal of the solvent under reduced pressure afforded a mixture of the isomeric diketones which were separated by chromatography (5% EtOAc/hexanes). (Compound 100C): 1H NMR ($CDCl_3$): δ 8.55 (1H, d, J=2.0 Hz), 8.13 (1H, dd, J=2.0, 8.3 Hz), 7.53 (1H, d, J=8.3 Hz), 2.77 (2H, t, J=6.6 Hz), 2.62 (3H, s), 2.05 (2H, t, J=6.6 Hz), 1.41 (6H, s). (Compound 100D): 1H NMR ($CDCl_3$): δ 8.10 (1H, d, J=8.1 Hz), 8.02 (1H, d, J=1.6 Hz), 7.82 (1H, dd, J=1.6, 8.1 Hz), 2.77 (2H, t, J=7.1 Hz), 2.64 (3H, s), 2.05 (2H, t, J=7.1 Hz), 1.44 (6H, s).

3,4-dihydro-4,4-dimethyl-6-(2-(2-methyl-1,3-dioxolanyl))-1(2H)-naphthalenone (Compound 100E)

A solution of 1.80 g (8.34 mmol) of a 1:5 mixture of 3,4-dihydro-4,4-dimethyl-7-acetyl-1(2H)- naphthalenone (Compound 100C); and 3,4-dihydro-4,4-dimethyl-6-acetyl-1(2H)-naphthalenone (Compound 100D) in 50 ml benzene was combined with 517.7 mg (8.34 mmol) of ethylene glycol and 20.0 mg (0.11 mmol) of p- toluenesulfonic acid monohydrate. The resulting solution was heated to reflux for 18 hours, cooled to room temperature, and concentrated under reduced pressure. The title compound was isolated by column chromatography (10% EtOAc-hexanes) as a colorless oil. 1H NMR ($CDCl_3$): δ 8.01 (1H, d, J=8.2 Hz), 7.51 (1H, s), 7.43 (1H, dd, J=1.7, 6.4 Hz), 4.07 (2H, m), 3.79 (2H, m), 2.74 (2H, t, J=6.5 Hz), 2.04 (2H, t, J=7.1 Hz), 1.67 (3H, s), 1.46 (6H, s).

1,2,3,4-tetrahydro-1-hydroxy-1-(4-methylphenyl)-4,4-dimethyl-6-(2-(2-methyl-1,3-dioxolanyl))naphthalene (Compound 100F)

To a solution of 496.2 mg (2.54 mmol) p- tolulylmagnesiumbromide in 20 ml THF (2.54 ml; 1M solution in ether) was added a solution of 3,4-dihydro-4,4-dimethyl-6-(2-(2-methyl-1,3-dioxolan- yl))-1(2H)-naphthalenone (Compound 100E, 200.0 mg, 0.769 mmol) in THF (5 ml). The solution was refluxed for 16 hours, cooled to room temperature, and washed with water, saturated aqueous NH_4Cl, and dried over $MgSO_4$. Removal of the solvents under reduced pressure and column chromatography (10% EtOAc/hexanes) afforded the title compound as a colorless solid. 1H NMR ($CDCl_3$): δ 7.49 (1H, d, J=1.7 Hz), 7.19 (2H, m), 7.10 (2H, d, J=7.9 Hz), 7.04 (1H, d, J=8.2 Hz), 4.05 (2H, m), 3.80 (2H, m), 2.34 (3H, s), 2.21 (1H, m), 2.10 (1H, m), 1.88 (1H, m), 1.65 (3H, s), 1.54 (1H, m), 1.39 (3H, s), 1.33 (3H, s).

3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-6-acetylnaphthalene (Compound 100G)

A solution of 1,2,3,4-tetrahydro-1-hydroxy-1-(4-methylphenyl)-4,4-dimethyl-6-(2-(2-methyl-1,3-dioxolanyl))naphthalene (Compound 100F 160.0 mg, 0.52 mmol), p-toluenesulfonic acid monohydrate (4 mg) and 30 ml benzene was refluxed for 12 hours. After cooling to room temperature, the reaction mixture was diluted with ether (100 ml) and washed with 10% aqueous sodium bicarbonate, water, and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and the solvents were removed under reduced pressure to give the title compound, which was isolated by column chromatography (10% EtOAc-hexanes) as a yellow oil. 1H NMR ($CDCl_3$): δ 7.97 (1H, d, J=1.8 Hz), 7.67 (1H, dd, J=1.7, 6.4 Hz), 7.22 (4H, s), 7.13 (1H, d, J=8.1 Hz), 6.10 (1H, t, J=4.5 Hz), 2.59 (3H, s), 2.40 (3H, s), 2.38 (2H, d, J=4.7 Hz), 1.38 (6H, s).

4-[3-oxo-3-(7.8-dihydro-5-(4-methylphenyl) -8,8-dimethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (Compound 101)

To a solution of 78.7 mg (0.272 mmol) 3,4-dihydro-1-(4-methylphenyl)-4,4-dimethyl-6-acetylnaphthalene (Compound 100G) in 4.0 ml of MeOH was added 53.1 mg (0.354 mmol) of 4-carboxy benzaldehyde, and 80. mg (2.00 mmol; 2.0 ml of 1M aqueous NaOH). The resulting solution was stirred at room temperature for 12 hours, concentrated under reduced pressure, and the residual oil dissolved in EtOAc. The solution was treated with 10% HCl, and the organic layer was washed with H_2O, and saturated aqueous NaCl, then dried over Na_2SO_4. Removal of the solvents under reduced pressure gave the title compound as a colorless solid which was purified by recrystallization from CH_3CN. 1H NMR (acetone-d6): δ 8.00 (7H, m), 7.83 (1H, d, J=15.6 Hz), 7.24 (4H, s), 7.13 (1H, d, J=8.1 Hz), 6.12 (1H, t, J=4.5 Hz), 2.42 (2H, d, J=4.8 Hz), 2.38 (3H, s), 1.41 (6H, s).

3,4-dihydro-1-phenyl-4,4-dimethyl-6-acetylnaphthalene (Compound 100H)

To a solution of 508.0 mg (1.95 mmol) of 3,4- dihydro-4,4-dimethyl-6-(2-(2-methyl-1,3-dioxolanyl))- 1(2H)-naphthalenone (Compound 100E) in 10 ml of THF was added 496.2 mg (2.54 mmol; 2.54 ml of a 1 M solution in Et2O) of phenylmagnesium bromide. The resulting solution was heated to reflux for 8 hours, H_2O was added and heating continued for 30 minutes. The THF was removed under reduced pressure and the aqueous residue was extracted with EtOAc. The combined organic layers were dried ($MgSO_4$), concentrated under reduced pressure, and the title compound isolated from the residue by column chromatography (10% EtOAc-hexanes) as a colorless oil. 1H NMR ($CDCl_3$): δ 7.97 (1H, d, J=1.8 Hz), 7.67 (1H, dd, J=2.1, 8.0 Hz), 7.34 (5H, m), 7.10 (1H, d, J=8.1 Hz), 6.12 (1H, d, J=4.6 Hz), 2.59 (3H, s), 2.39 (2H, d, J=4.8 Hz), 1.38 (6H, s). 4-[3 -oxo-3-(7,8-dihydro-5-phenyl-8,8-dimethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (Compound 103)

To a solution of 115.0 mg (0.42 mmol) of 3,4- dihydro-1-phenyl-4,4-dimethyl-6-acetylnaphthalene (Compound 100H) and 65.0 mg (0.43 mmol) of 4-formyl- benzoic acid in 5.0 ml EtOH and 1.0 ml THF, was added 120.0 mg (3.00 mmol; 3.0 ml of a 1 M aqueous solution) of NaOH. The resulting yellow solution was stirred at room temperature for 12 hours. The solution was acidified with 6% aqueous HCl and extracted with EtOAc. The combined organic layers were dried ($MgSO_4$), concentrated under reduced pressure, and the title compounds was isolated by column chromatography (50% EtOAc-hexanes) as a pale yellow solid. 1H NMR ($CDCl_3$): δ 8.13 (2H, d, J=7.7 Hz), 8.04 (1H, s), 7.81 (1H, d, J=15.5 Hz), 7.75 (3H, m), 7.60 (1H, d, J=15.5 Hz), 7.35 (5H, m), 7.14 (1H, d, J=8.1 Hz), 6.15 (1H, t, J=4.2 Hz), 2.41 (2H, d, J=4.2 Hz), 1.41 (6H, s).

Method of Potentiating Nuclear Receptor Agonists

Overview and introduction

We have discovered that a subset of retinoid antagonists which exhibit negative hormone activity can be used for potentiating the biological activities of other retinoids and steroid receptor superfamily hormones. These other retinoids and steroid receptor superfamily hormones can be either endogenous hormones or pharmaceutical agents. Thus, for example, when used in combination with a retinoid negative hormone, certain activities of pharmaceutical retinoid agonists can be rendered more active in eliciting specific biological effects. Advantageously, this combination approach to drug administration can minimize undesirable side effects of pharmaceutical retinoids because lower dosages of the pharmaceutical retinoids can be used with improved effectiveness.

More particularly, we have discovered that AGN 193109, a synthetic retinoid having the structure shown in FIG. 1, exhibits unique and unexpected pharmacologic activities. AGN 193109 exhibits high affinity for the RAR subclass of nuclear receptors without activating these receptors or stimulating transcription of retinoid responsive genes. Instead, AGN 193109 inhibits the activation of RARs by retinoid agonists and therefore behaves as a retinoid antagonist.

Additionally, we have discovered that retinoid negative hormones can be used without coadministration of a retinoid agonist or steroid hormone to control certain disease symptoms. More specifically, the retinoid negative hormone disclosed herein can down-regulate the high level basal transcription of genes that are responsive to unliganded RARs. If, for example, uncontrolled cellular proliferation results from the activity of genes responsive to unliganded RARs, then that gene activity can be reduced by the administration of a retinoid negative hormone that inactivates RARs. Consequently, cellular proliferation dependent on the activity of unliganded RARs can be inhibited by the negative hormone. Inhibition of unliganded RARs cannot be achieved using conventional antagonists.

Significantly, we have discovered that AGN 193109 can both repress RAR basal activity and can sometimes potentiate the activities of other retinoid and steroid receptor superfamily hormone agonists. In the context of the invention, a hormone agonist is said to be potentiated by a negative hormone such as AGN 193109 if, in the presence of the negative hormone, a reduced concentration of the agonist elicits substantially the same quantitative response as that obtainable with the agonist alone. The quantitative response can, for example, be measured in a reporter gene assay in vitro. Thus, a therapeutic retinoid that elicits a desired response when used at a particular dosage or concentration is potentiated by AGN 193109 if, in combination with AGN 193109, a lower dosage or concentration of the therapeutic retinoid can be used to produce substantially the same effect as a higher dosage or concentration of the therapeutic retinoid when that therapeutic retinoid is used alone. The list of agonists that can be potentiated by coadministration with AGN 193109 includes RAR agonists, vitamin D receptor agonists, glucocorticoid receptor agonists and thyroid hormone receptor agonists. More particularly, specific agonists that can be potentiated by coadministration include: ATRA, 13-cis retinoic acid, the synthetic RAR agonist AGN 191183, 1,25-

dihydroxyvitamin D_3, dexamethasone and thyroid hormone (3,3',5-triiodothyronine). Also disclosed herein is a method that can be used to identify other hormones that can be potentiated by coadministration with AGN 193109.

Thus, AGN 193109 behaves in a manner not anticipated for a simple retinoid antagonist, but as a negative hormone that can potentiate the activities of various members of the family of nuclear receptors. We also disclose a possible mechanism that can account for both negative hormone activity and the ability of AGN 193109 to potentiate the activities of other nuclear receptor ligands. This mechanism incorporates elements known to participate in retinoid-dependent signalling pathways and additionally incorporates a novel negative regulatory component.

Those having ordinary skill in the art will appreciate that RARs, which are high affinity targets of AGN 193109 binding, are transcription factors that regulate the expression of a variety of retinoid responsive genes. Cis-regulatory DNA binding sites for the RARs have been identified nearby genes that are transcriptionally regulated in a retinoid-dependent fashion. RAR binding to such DNA sites, known as retinoic acid response elements (RAREs), has been well defined. Importantly, the RAREs bind heterodimers consisting of one RAR and one RXR. The RXR component of the heterodimer functions to promote a high affinity interaction between the RAR/RXR heterodimer and the RARE (Mangelsdorf et al. *The Retinoid Receptors* in *The Retinoids: Biology. Chemistry and Medicine,* 2nd edition, eds. Sporn et al., Raven Press, Ltd., New York 1994, the disclosure of which is hereby incorporated by reference).

As detailed below, our findings related to the negative hormone activity of AGN 193109 are consistent with a mechanism involving the interaction of a putative Negative Coactivator Protein (NCP) with the RAR. According to the proposed mechanism, this interaction is stabilized by AGN 193109.

Our results further indicated that AGN 193109 can modulate intracellular availability of NCP for interaction with nuclear receptors other than RARs that are occupied by AGN 193109. It follows that AGN 193109 can potentiate transcriptional regulatory pathways involving nuclear receptors that share with the RARs the ability to bind the NCP. In this regard, AGN 193109 exhibits the ability to modulate a variety of nuclear receptor pathways, an activity that would not be predicted for a conventional retinoid antagonist. Accordingly, AGN 193109 is useful as an agent for potentiating the activity of nuclear receptor ligands, including both endogenous hormones and prescribed therapeutics. This specific embodiment illustrates the more general principle that any nuclear receptor negative hormone will potentiate the activity of other nuclear receptors that competitively bind the NCP.

Although other materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. General references for methods that can be used to perform the various nucleic acid manipulations and procedures described herein can be found in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) and *Current Protocols in Molecular Biology* (Ausubel et al. eds., Greene Publishing Associates and Wiley-Interscience 1987). The disclosures contained in these references are hereby incorporated by reference. A description of the experiments and results that led to the creation of the present invention follows.

Example 6 describes the methods used to demonstrate that AGN 193109 bound each of three RARs with high affinity but failed to activate retinoid dependent gene expression.

EXAMPLE 6

AGN 193109 Binds RARs With High Affinity But Does Not Transactivate Retinoid-Dependent Gene Expression

Human RAR-α, RAR-β and RAR-γ receptors were separately expressed as recombinant proteins using a baculovirus expression system essentially according to the method described by Allegretto et al. in *J Biol. Chem.* 268:26625 (1993). The recombinant receptor proteins were separately employed for determining AGN 193109 binding affinities using the [^{3}H]-ATRA displacement assay described by Heyman et al. in *Cell* 68:397 (1992). Dissociation constants (Kds) were determined according to the procedure described by Cheng et al. in *Biochemical Pharmacology* 22:3099 (1973).

AGN 193109 was also tested for its ability to transactivate RARs in CV-1 cells transiently cotransfected with RAR expression vectors and a retinoid responsive reporter gene construct. Receptor expression vectors pRShRAR-α (Giguere et al. *Nature* 330:624 (1987)), pRShRAR-β (Benbrook et al. *Nature* 333:669 (1988)) and pRShRAR-γ (Ishikawa et al. *Mol. Endocrinol.* 4:837 (1990)) were separately cotransfected with the ΔMTV-TREp-Luc reporter plasmid. Use of this luciferase reporter plasmid has been disclosed by Heyman et al. in *Cell* 68:397 (1992). The ΔMTV-TREp-Luc plasmid is essentially identical to the ΔMTV-TREp-CAT reporter construct described by Umesono et al. in *Nature* 336:262 (1988), except that the chloramphenicol acetyltransferase (CAT) reporter gene was substituted by a polynucleotide sequence encoding firefly luciferase. Transfection of green monkey CV-1 cells was carried out using the calcium phosphate coprecipitation method described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989). CV-1 cells were plated at a density of 4×10^4/well in 12 well multiwell plates and transiently transfected with a calcium phosphate precipitate containing 0.7 μg of reporter plasmid and 0.1 μg of receptor plasmid according to standard laboratory procedures. Cells were washed after 18 hours to remove the precipitate and refed with Dulbecco's modified Eagle's medium (DMEM) (Gibco), containing 10% activated charcoal extracted fetal bovine serum (Gemini Bio-Products). Cells were treated with vehicle alone (ethanol) or AGN 193109 (10^{-9} to 10^{-6} M) for 18 hours. Cell lysates were prepared in 0.1 M KPO_4 (pH 7.8), 1.0% TRITON X-100, 1.0 mM DTT, 2 mM EDTA. Luciferase activity was measured as described by de Wet et al. in *Mol. Cell. Biol* 7:725 (1987), using firefly luciferin (Analytical Luminescence Laboratory) and an EG&G Berthold 96-well plate luminometer. Reported luciferase values represented the mean ± SEM of triplicate determinations.

The results presented in Table 11 indicated that AGN 193109 bound each of RAR-α, RAR-β and RAR-γ with high affinity, but did not activate retinoid-dependent gene expression. More specifically, AGN 193109 bound each of the three receptors with Kd values in the 2–3 nM range. Despite this tight binding, AGN 193109 failed to activate gene expression when compared with inductions stimulated by ATRA. Accordingly, the half-maximal effective concentration of AGN 193109 (EC_{50}) was unmeasurable. Although not presented in the Table, we also found that AGN 193109 had no measurable affinity for the RXRs.

TABLE 11

AGN 193109 Binding and Transactivation of the RARs

	RAR-α	RAR-β	RAR-γ
EC_{50} (nM)	No Activity	No Activity	No Activity
K_d (nM)	2	2	3

Example 7 describes the methods used to demonstrate that AGN 193109 is an antagonist of ATRA dependent gene expression.

EXAMPLE 7

AGN 193109-Dependent Inhibition of RAR Transactivation by ATRA

The ability of AGN 193109 to antagonize ATRA mediated RAR activation was investigated in CV-1 cells cotransfected by the calcium phosphate coprecipitation method of Sambrook et al. (*Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Lab Publ. 1989). Eukaryotic expression vectors pRShRAR-α (Giguere et al. *Nature* 330:624 (1987)), pRShRAR-β (Benbrook et al. *Nature* 333:669 (1988)) and pRShRAR-γ (Ishikawa et al. *Mol. Endocrinol.* 4:837 (1990)) were cotransfected with the Δ-MTV-Luc reporter plasmid described by Hollenberg et al. (*Cell* 55:899 (1988)). Notably, the reporter plasmid contained two copies of the TRE-palindromic response element. Calcium phosphate transfections were carried out exactly as described in Example 6. Cells were dosed with vehicle alone (ethanol), ATRA (10^{-9} to 10^{-6} M), AGN 193109 (10^{-9} to 10^{-6} M), or 10^{-8} M ATRA in combination with AGN 193109 (10^{-9} to 10^{-6} M) for 18 hours. Cell lysates and luciferase activity measurements were also performed as in Example 6.

The results of these procedures are presented in FIGS. 2A through 2F where luciferase values represent the mean ±SEM of triplicate determinations. More specifically, the results presented in FIGS. 2A, 2C and 2E indicated that stimulation of transfected cells with ATRA led to dose responsive increases in luciferase activity. This confirmed that ATRA activated each of the three RARs in the experimental system and provided a comparative basis for detecting the activity of an antagonist. The graphic results presented in FIGS. 2B, 2D and 2F indicated that cotreatment of transfected cells with 10 nM ATRA and increasing concentrations of AGN 193109 led to an inhibition of luciferase activity. In particular, equal doses of AGN 193109 and ATRA gave greater than 50% inhibition relative to ATRA alone for all three RAR subtypes. Comparison of the ATRA dose response in the presence of different concentrations of AGN 193109 indicated that ATRA was competitively inhibited by AGN 193109. Notably, the horizontal axes on all of the graphs shown in FIG. 2 represents the log of the retinoid concentration. These results proved that AGN 193109 was a potent RAR antagonist.

We next performed experiments to elucidate the mechanism underlying the antagonist activity of AGN 193109. Those having ordinary skill in the art will appreciate that nuclear receptor activation is believed to involve a conformational change of the receptor that is induced by ligand binding. Indeed, the results of protease protection assays have confirmed that nuclear hormone agonists and antagonists cause receptor proteins to adopt different conformations (Keidel et al. Mol. *Cell. Biol.* 14:287 (1994); Allan et al. *J. Biol. Chem.* 267:19513 (1992)). We used such an assay to determine if AGN 193109 and ATRA caused RAR-α to adopt different conformations. AGN 193583, an RAR-α-selective antagonist, was included as a positive control that is known to confer an antagonist-specific pattern of protease sensitivity.

Example 8 describes one method that was used to detect conformational changes in RAR-α resulting from AGN 193109 binding. As presented below, the results of this procedure unexpectedly indicated that AGN 193109 led to a pattern of trypsin sensitivity that was substantially identical to that induced by ATRA, an RAR agonist, and unlike that induced by a model RAR antagonist. This finding suggested that AGN 193109 possessed properties distinct from other retinoid antagonists.

EXAMPLE 8

Protease Protection Analysis

A plasmid constructed in the vector pGEM3Z (Pharmacia) and containing the RAR-α cDNA (Giguere et al. *Nature* 330:624 (1987)), was used in connection with the TNT-coupled reticulocyte lysate in vitro transcription-translation system (Promega) to prepare [^{35}S]-methionine labeled RAR-α. Limited proteolytic digestion of the labeled protein RAR-α was carried out according to the method described by Keidel et al. in *Mol. Cell. Biol.* 14:287 (1994). Aliquots of reticulocyte lysate containing [^{35}S]-methionine labeled RAR-α were incubated with either ATRA, AGN 193583 or AGN 193109 on ice for 45 minutes in a total volume of 9 μl. The retinoid final concentration for all trials was 100 nM for ATRA and AGN 193109, and 1000 nM for AGN 193583. The difference between the final concentrations of the retinoids was based on the approximate 10-fold difference in relative affinities of ATRA and AGN 193109 (having Kd at RAR-α of 2 and 10 nM, respectively) and AGN 193583 (having Kd at RAR-α of ≧100 nM). After ligand binding, 1 μl of appropriately concentrated trypsin was added to the mixture to give final concentrations of 25, 50 or 100 μg/ml. Samples were incubated at room temperature for 10 minutes and trypsin digestion stopped by addition of SDS-sample buffer. Samples were subjected to polyacrylamide gel electrophoresis and autoradiographed according to standard procedures.

Both the agonist and antagonist led to distinct patterns of trypsin sensitivity that were different from the result obtained by digestion of the unliganded receptor. Autoradiographic results indicated that trypsin concentrations of 25, 50 and 100 μg/ml completely digested the radiolabeled RAR-α in 10 minutes at room temperature in the absence of added retinoid. Prebinding of ATRA led to the appearance of two major protease resistant species. Prebinding of the RAR-α-selective antagonist AGN 193583 gave rise to a protease resistant species that was of lower molecular weight than that resulting from ATRA prebinding. This result demonstrated that a retinoid agonist and antagonist led to conformational changes detectable by virtue of altered trypsin sensitivities. Surprisingly, prebinding of AGN 193109 gave rise to a protease protection pattern that was indistinguishable from that produced by prebinding of ATRA.

The results presented above confirmed that AGN 193109 bound the RAR-α and altered its conformation. Interestingly, the nature of this conformational change more closely resembled that which resulted from binding of an agonist (ATRA) than the alteration produced by antagonist (AGN 193583) binding. Clearly, the mechanism of AGN 193109 dependent antagonism was unique.

We considered possible mechanisms that could account for the antagonist activity of AGN 193109. In particular, we used a standard gel shift assay to test whether AGN 193109 perturbed RAR/RXR heterodimer formation or inhibited the interaction between RAR and its cognate DNA binding site.

Example 9 describes a gel electrophoretic mobility-shift assay used to demonstrate that AGN 193109 neither inhibited RAR/RXR dimerization nor inhibited binding of dimers to a target DNA.

EXAMPLE 9

Gel Shift Analysis

In vitro translated RAR-α was produced essentially as described under Example 8, except that ^{35}S-labeled methoinine was omitted. In vitro translated RXR-α was similarly produced using a pBluescript(II)(SK)-based vector containing the RXR-α cDNA described by Mangelsdorf, et al. in *Nature* 345:224–229 (1990) as the template for generating in vitro transcripts. The labeled RAR-α and RXR-α, alone or in combination, or prebound with AGN 193109 (10^{-6} M) either alone or in combination, were allowed to interact with an end-labeled DR-5 RARE double-stranded probe having the sequence 5'-TCAGGTCACCAGGAGGTCAGA-3' (SEQ ID NO:1). The binding mixture was electrophoresed on a non-denaturing polyacrylamide gel and autoradiographed according to standard laboratory procedures. A single retarded species appearing on the autoradiograph that was common to all the lanes on the gel represented an undefined probe-binding factor present in the reticulocyte lysate. Only the RAR/RXR combination gave rise to a retinoid receptor-specific retarded species. Neither RAR alone nor RXR alone bound the probe to produce this shifted species. The presence of AGN 193109 did not diminish this interaction.

These results indicated that AGN 193109 did not substantially alter either the homo- or hetero-dimerization properties of RAR-α. Further, AGN 193109 did not inhibit the interaction of receptor dimers with a DNA segment containing the cognate binding site.

In view of the unique properties which characterized AGN 193109, we proceeded to investigate whether this antagonist could additionally inhibit the activity of unliganded RARs. The receptor/reporter system used to make this determination advantageously exhibited high level constitutive activity in the absence of added retinoid agonist. More specifically, these procedures employed the ER-RAR chimeric receptor and ERE-tk-Luc reporter system. The ERE-tk-Luc plasmid includes the region −397 to −87 of the estrogen responsive 5'-flanking region of the Xenopus vitellogenin A2 gene, described by Klein-Hitpass, et al. in *Cell* 46:1053–1061 (1986), ligated upstream of the HSV thymidine kinase promoter and luciferase reporter gene of plasmid tk-Luc. The ER-RAR chimeric receptors consisted of the estrogen receptor DNA binding domain fused to the "D-E-F" domain of the RARs. Those having ordinary skill in the art appreciate this "D-E-F" domain functions to bind retinoid, to provide a retinoid inducible transactivation function and to provide a contact site for heterodimerization with RXR. Thus, luciferase expression in this reporter system was dependent on activation of the transfected chimeric receptor construct.

Example 10 describes the method used to demonstrate that AGN 193109 inhibited basal gene activity attributable to unliganded RARs. These procedures were performed in the absence of added retinoid agonist. The results presented below provided the first indication that AGN 193109 exhibited negative hormone activity.

EXAMPLE 10

Repression of Basal Gene Activity of a Retinoid-Regulated Reporter in Transiently Cotransfected Cell Lines

CV-1 cells were co-transfected with the ERE-tk-Luc reporter plasmid and either ER-RAR-α, ER-RAR-β or ER-RAR-γ expression plasmids. The ERE-tk-Luc plasmid contained the estrogen-responsive promoter element of the *Xenopus laevis* vitellogenin A2 gene and was substantially identical to the reporter plasmid described by Klein-Hitpass et al. in *Cell* 46:1053 (1986), except that the CAT reporter gene was substituted by a polynucleotide sequence encoding luciferase. The ER-RAR-α, ER-RAR-β and ER-RAR-γ chimeric receptor-encoding polynucleotides employed in the co-transfection have been described by Graupner et al. in *Biochem. Biophys. Res. Comm.* 179:1554 (1991). These polynucleotides were ligated into the pECE expression vector described by Ellis et al. in *Cell* 45:721 (1986) and expressed under transcriptional control of the SV-40 promoter. Calcium phosphate transfections were carried out exactly as described in Example 6 using 0.5 μg/well of reporter plasmid and either 0.05 μg, 0.10 μg or 0.2 μg/well of receptor plasmid. Cells were dosed with vehicle alone (ethanol), ATRA (10^{-9} to 10^{-6} M), or AGN 193109 (10^{-9} to 10^{-6} M) for 18 hours. Cell lysates and luciferase activity measurements were performed as described in Example 6.

The results presented in FIGS. **3A**, **4A** and **5A** confirmed that ATRA strongly induced luciferase expression in all transfectants. Basal level expression of luciferase for the three transfected chimeric RAR isoforms ranged from approximately 7,000 to 40,000 relative light units (rlu) and was somewhat dependent on the amount of receptor plasmid used in the transfection. Thus, the three chimeric receptors were activatable by ATRA, as expected. More specifically, all three receptors bound ATRA and activated transcription of the luciferase reporter gene harbored on the ERE-tk-Luc plasmid.

FIGS. **3B**, **4B** and **5B** present AGN 193109 dose response curves obtained in the absence of any exogenous retinoid agonist. Interestingly, ER-RAR-α (FIG. **3B**) was substantially unaffected by AGN 193109, while the ER-RAR-β and ER-RAR-γ chimeric receptors (FIGS. **4B** and **5B**, respectively) exhibited an AGN 193109 dose responsive decrease in luciferase reporter activity.

We further investigated the negative hormone activity of AGN 193109 by testing its ability to repress gene expression mediated by a chimeric RAR-γ receptor engineered to possess a constitutive transcription activator domain. More specifically, we used a constitutively active RAR-γ chimeric receptor fused to the acidic activator domain of HSV VP-16, called RAR-γ-VP-16, in two types of luciferase reporter systems. The first consisted of the ERE-tk-Luc reporter cotransfected with ER-RARs and ER-RXR-α. The second utilized the αMTV-TREp-Luc reporter instead of the ERE-tk-Luc reporter.

Example 11 describes the method used to demonstrate that AGN 193109 could suppress the activity of a transcription activator domain of an RAR. The results presented below proved that AGN 193109 could suppress RAR-dependent gene expression in the absence of an agonist and confirmed that AGN 193109 exhibited negative hormone activity.

EXAMPLE 11

Repression of RAR-VP-16 Activity in Transiently Transfected Cells

CV-1 cells were transiently cotransfected according to the calcium phosphate coprecipitation technique described under Example 6 using 0.5 μg/well of the ERE-tk-Luc luciferase reporter plasmid, 0.1 μg/well of the ER-RXR-α chimeric reporter expression plasmid, and either 0 μg or 0.1 μg/well of the RAR-γ-VP-16 expression plasmid. The chimeric receptor ER-RXR-α consisted of the hormone binding domain (amino acids 181 to 458) of RXR-α (Mangelsdorf, et al. *Nature* 345:224–229 (1990)) fused to the estrogen receptor DNA binding domain (Graupner, et al. *Biochem. Biophys. Res. Comm.* 179:1554 (1991)) and was expressed from the SV-40 based expression vector pECE described by Ellis, et al. in *Cell* 45:721 (1986). RAR-γ-VP-16 is identical to the VP16RAR-γl expression plasmid described by Nagpal et al. in *EMBO J.* 12:2349 (1993), the disclosure of which is hereby incorporated by reference, and encodes a chimeric protein having the activation domain of the VP-16 protein of HSV fused to the amino-terminus of full length RAR-γ. At eighteen hours post-transfection, cells were rinsed with phosphate buffered saline (PBS) and fed with DMEM (Gibco-BRL) containing 10% FBS (Gemini Bio-Products) that had been extracted with charcoal to remove retinoids. Cells were dosed with an appropriate dilution of AGN 193109 or ATRA in ethanol vehicle or ethanol alone for 18 hours, then rinsed with PBS and lysed using 0.1 M KPO_4 (pH 7.8), 1.0% TRITON X-100, 1.0 mM DTT, 2 mM EDTA. Luciferase activity was measured according to the method described by de Wet, et al. in *Mol. Cell. Biol.* 7:725 (1987), using firefly luciferin (Analytical Luminescence Laboratory) and an EG&G Berthold 96-well plate luminometer. Luciferase values represented the mean ±SEM of triplicate determinations.

As shown in FIG. 6, CV-1 cells transfected with the ERE-tk-Luc reporter construct and the ER-RAR-α chimeric expression plasmid exhibited a weak activation of luciferase activity by ATRA, likely due to isomerization of ATRA to 9C-RA, the natural ligand for the RXRs (Heyman et al. *Cell* 68:397 (1992). Cells transfected with the same mixture of reporter and chimeric receptor plasmids but treated with AGN 193109 did not exhibit any effect on luciferase activity. As AGN 193109 does not bind to the RXRs, this latter result was expected. CV-1 cells similarly transfected with the ERE-tk-Luc reporter but with substitution of an ER-RAR chimeric receptor expression plasmid for ER-RXR-α exhibited a robust induction of luciferase activity following ATRA treatment.

In contrast, inclusion of the RAR-γ-VP-16 expression plasmid with the ER-RXR-α and ERE-tk-Luc plasmids in the transfection mixture resulted in a significant increase in the basal luciferase activity as measured in the absence of any added retinoid. This increase in basal luciferase activity observed for the ER-RXR-α/RAR-γ-VP-16 cotransfectants, when compared to the result obtained using cells transfected with ER-RXR-α alone, indicated that recombinant ER-RXR-α and RAR-γ-VP-16 proteins could heterodimerize. Interaction of the heterodimer with the cis-regulatory estrogen responsive element led to a targeting of the VP-16 activation domain to the promoter region of the ERE-tk-Luc reporter. Treatment of such triply transfected cells with ATRA led to a modest increase of luciferase activity over the high basal level. However, treatment of the triple transfectants with AGN 193109 resulted in a dose dependent decrease in luciferase activity. Importantly, FIG. 6 shows that AGN 193109 treatment of cells cotransfected with ER-RXR-α and RAR-γ-VP-16 led to repression of luciferase activity with maximal inhibition occurring at approximately 10^{-8} M AGN 193109.

Our observation that AGN 193109 repressed the constitutive transcriptional activation function of RAR-γ-VP-16 in the presence of an RXR was explained by a model wherein binding of AGN 193109 to the RAR induced a conformational change in the RAR which stabilizes a negative conformation that facilitates the binding of a trans-acting negative coactivator protein. When the AGN 193109/RAR complex is bound by the NCP, the RAR is incapable of upregulating transcription of genes that are ordinarily responsive to activated RARs. Our model further proposes that the intracellular reservoir of NCP is in limiting concentration in certain contexts and can be depleted by virtue of AGN 193109 stimulated complexation with RARs.

The results presented in FIG. 6 additionally indicated that even at 10^{-6} M AGN 193109, the ER-RXR-α and RAR-γ-VP-16 proteins could interact to form heterodimers competent for activating transcription of the reporter gene. More specifically, cells transfected with ER-RXR-α and RAR-γ-VP-16 and treated with AGN 193109 at a concentration (10^{-8}–10^{-6} M) sufficient to provide maximal inhibition, gave luciferase activity readings of approximately 16,000 rlu. Conversely, cells transfected only with ER-RXR-α and then treated with AGN 193109 at a concentration as high as 10^{-6} M exhibited luciferase expression levels of only approximately 8,000 rlu. The fact that a higher level of luciferase activity was obtained in cells that expressed both ER-RXR-α and RAR-γ-VP-16, even in the presence of 10^{-6} M AGN 193109 demonstrated the persistence of an interaction between the two recombinant receptors. The repression of RAR-γ-VP-16 activity by AGN 193109 suggested that modulation of NCP interaction can be codominate with VP-16 activation. Accordingly, we realized that it may be possible to modulate the expression of genes which are not ordinarily regulated by retinoids in an AGN 193109 dependent manner.

Candidates for AGN 193109 regulatable genes include those that are activated by transcription factor complexes which consist of non-RAR factors that associate or heterodimerize with RARs, wherein the non-RAR factor does not require an RAR agonist for activation. While stimulation with an RAR agonist may have substantially no effect on the expression of such genes, administration with AGN 193109 can promote formation of inactive transcription complexes comprising AGN 193109/RAR/NCP. Consequently, addition of the AGN 193109 retinoid negative hormone can down-regulate transcription of an otherwise retinoid-insensitive gene.

This same mechanism can account for the observation that AGN 193109 can repress the activity of the tissue transglutaminase (TGase) gene in HL-60 cells. A retinoid response element consisting of three canonical retinoid half sites spaced by 5 and 7 base pairs has been identified in the transcription control region of this gene. While TGase can be induced by RXR-selective agonists, it is not responsive to RAR-selective agonists. The TGase retinoid response element is bound by an RAR/RXR heterodimer (Davies et al. in Press). Interestingly, AGN 193109 is able to repress TGase activity induced by RXR agonists. This AGN 193109 mediated repression can be accounted for by the ability of this negative hormone to sequester NCPs to the RAR component of the heterodimer, thereby repressing the activity of the associated RXR.

We have also obtained results which support conclusions identical to those presented under Example 11 by employing

RAR-γ-VP-16 and expression constructs and the ΔMTV-TREp-Luc reporter plasmid instead of the RAR-γ-VP-16 and ER-RXR-α expression constructs in combination with the ERE-tk-Luc reporter plasmid. Consistent with the results presented above, we found that RAR-γ-VP-16 activity at the ΔMTV-TREp-Luc reporter was inhibited by AGN 193109. Therefore, AGN 193109 repressed RAR-γ-VP-16 activity when this chimeric receptor was directly bound to a retinoic acid receptor response element instead of indirectly bound to an estrogen response element in the promoter region of the reporter plasmid. These findings demonstrated that an assay for identifying agents having negative hormone activity need not be limited by the use of a particular reporter plasmid. Instead, the critical feature embodied by an experimental system useful for identifying retinoid negative hormones involves detecting the ability of a compound to repress the activity of an RAR engineered to contain a constitutive transcription activation domain.

Generally, retinoid negative hormones can be identified as the subset of retinoid compounds that repress within a transfected cell the basal level expression of a reporter gene that is transcriptionally responsive to direct or indirect binding by a retinoid receptor or a chimeric receptor that includes at least the domains of the retinoid receptor located C-terminal to the DNA binding domain of that receptor. This approach has been adapted to a screening method useful for identifying retinoid negative hormones. In the various embodiments of the invented screening method, the structure of the receptor for which a negative hormone is sought is variable. More specifically, the retinoid receptor can be either of the RAR or the RXR subtype. The receptor can optionally be engineered to include a constitutive transcription activator domain. The retinoid receptor used to screen for negative hormones optionally contains a heterologous DNA binding domain as a substitute for the DNA binding domain endogenous to the native receptor. However, when a second receptor is used in the screening method, and where the second receptor can dimerize with the retinoid receptor for which a negative hormone is sought, then that retinoid receptor may not require a DNA binding domain because it can be linked to the transcription control region of the reporter gene indirectly through dimerization with the second receptor which is itself bound to the transcription control region.

In the practice of the screening method, the ability of a compound to repress the basal expression of a reporter is typically measured in an in vitro assay. Basal expression represents the baseline level of reporter expression in transfected cells under conditions where no exogenously added retinoid agonist is present. Optionally, steps may be taken to remove endogenous retinoid ligands from the environment of the transfected cells via procedures such as charcoal extraction of the serum that is used to culture cells in vitro.

Examples of reporter genes useful in connection with the screening method include those encoding luciferase, beta galactosidase, chloramphenicol acetyl transferase or cell surface antigens that can be detected by immunochemical means. In practice, the nature of the reporter gene is not expected to be critical for the operability of the method. However, the transcriptional regulatory region of the reporter construct must include one or more cis-regulatory elements that are targets of transcription factors for which negative hormones are being sought. For example, if one desires to identify RAR negative hormones, then the transcriptional regulatory region of the reporter construct could contain a cis-regulatory element that can be bound by an RAR-containing protein. In this example, there should be correspondence between the DNA binding domain of the RAR and the cis-regulatory element of the transcriptional regulatory region of the reporter construct. Thus, if a chimeric RAR having a constitutive transcription activator domain and a DNA binding domain that can bind cis-regulatory estrogen responsive elements is employed in the screening method, then the transcriptional regulatory region of the reporter construct should contain an estrogen responsive element.

Examples of cis-regulatory elements that directly bind retinoid receptors (RAREs) useful in connection with the reporter assay are disclosed by Mangelsdorf et al. in *The Retinoid Receptors* in *The Retinoids: Biology, Chemistry and Medicine,* 2nd edition, eds. Sporn et al., Raven Press, Ltd., New York (1994), the disclosure of which has been incorporated by reference hereinabove. Examples of cis-regulatory elements that indirectly bind chimeric receptors include DNA binding sites for any DNA binding protein for which the DNA binding domain of the protein can be incorporated into a chimeric receptor consisting of this DNA binding domain attached to a retinoid receptor. Specific examples of heterologous DNA binding domains that can be engineered into chimeric receptors and that will recognize heterologous cis-regulatory elements include those recognizing estrogen responsive elements. Thus, the retinoid receptor portion of a chimeric receptor useful in connection with the screening method need not contain the DNA binding of the retinoid receptor but must contain at least the ligand binding domain of the retinoid receptor.

A further example of indirect retinoid receptor binding to the cis-regulatory element includes the use of a protein that can bind the cis-regulatory element and dimerize with a retinoid receptor. In this case, the retinoid receptor associates with the cis-regulatory element only by association with the protein responsible for DNA binding. An example of such a system would include the use of a fusion protein consisting of a heterologous DNA binding domain fused to an RXR, containing at least the domain of the RXR responsible for dimerization with RARs. Cointroduced RARs can dimerize with such a fusion protein bound to the cis-regulatory element. We anticipate that any cis-regulatory element-binding protein that dimerizes with RARs to result in an indirect association of the RAR with the cis-regulatory element will also be suitable for use with the negative hormone screening method.

In a preferred embodiment of the screening method, retinoid negative hormones are identified as those retinoids that repress basal expression of an engineered RAR transcription factor having increased basal activity. Although not essential for operability of the screening method, the engineered RAR employed in the following Example included a constitutive transcription activating domain. Use of this chimeric receptor advantageously provided a means by which the basal expression of a reporter gene could be elevated in the absence of any retinoid. Although we have employed transient transfection in the procedures detailed above, stably transfected cell lines constitutively expressing the chimeric receptor would also be useful in connection with the screening method.

As disclosed in the following Example, a chimeric retinoid receptor having a constitutive transcription activator domain was heterodimerizable with a second receptor engineered to contain a DNA binding domain specific for an estrogen responsive cis-regulatory element. In this case the chimeric retinoid receptor having a constitutive transcription activator domain associates with the cis-regulatory region controlling reporter gene expression indirectly via binding to

a second receptor that binds a DNA target sequence. More particularly, the second receptor was engineered to contain a DNA binding domain that recognized an estrogen responsive element. Advantageously, the reporter gene having an estrogen responsive element in the upstream promoter region was unresponsive to retinoid agonists in the absence of the transfected chimeric receptor having the constitutive transcription activator domain. Accordingly, all reporter gene activity was attributed to the transfected receptors. The combination use of the estrogen responsive element DNA binding domain and the estrogen responsive element cis-regulatory element are intended to be illustrative only. Those having ordinary skill in the art will realize that other combinations of engineered receptors having specificity for non-RARE cis-regulatory elements will also be useful in the practice of the invented screening method.

Cells useful in connection with the screening method will be eukaryotic cells that can be transfected. The cells may be animal cells such as human, primate or rodent cells. We have achieved very good results using CV-1 cells, but reasonably expect that other cultured cell lines could also be used successfully. Any of a number of conventional transfection methods known in the art can be used to introduce an expression construct encoding the chimeric retinoid receptor having a constitutive transcription activator domain.

The constitutive transcription activator domain will consist of a plurality of amino acids which will likely have an overall acidic character as represented by a negative charge under neutral pH conditions. For example, the constitutive transcription activator domain may have an amino acid sequence which is also found in viral transcription factors. One example of a viral transcription factor having a constitutive transcription activator domain is the herpes simplex virus 16. However, other viral or synthetic transcription activator domains would also be useful in the construction of expression constructs encoding the chimeric retinoid receptor having a constitutive transcription activator domain.

As described below, we have developed a generalized screening method useful for identifying retinoid negative hormones. This screening method provides a means for distinguishing simple antagonists from negative hormones. Table 12 lists several retinoid compounds which exhibit potent affinity for RAR-γ yet, with the exception of ATRA, did not transactivate this receptor in a transient cotransfection transactivation assay. We therefore tested these compounds to determine which were RAR-γ antagonists and which, if any, of these antagonists exhibited negative hormone activity.

Example 12 describes the method used to identify retinoid compounds that were antagonists, and the subset of antagonists that exhibited negative hormone activity.

EXAMPLE 12

Assay for Retinoid Negative Hormones

4×10^4 CV-1 cells were transfected by the calcium phosphate coprecipitation procedure described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) using 0.5 μg ERE-tk-Luc reporter plasmid and 0.1 μg ER-RAR-γ (Graupner et al. *Biochem. Biophys. Res. Comm.* 179:1554 (1991)) chimeric expression plasmid. After 18 hours, cells were rinsed with PBS and fed with DMEM (Gibco-BRL) containing 10% activated charcoal extracted FBS (Gemini Bio-Products). Cells were treated with 10^{-8} M ATRA in ethanol or ethanol alone. In addition, ATRA treated cells were treated with 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M of the compounds listed in Table 12. After 18 hours, cells were rinsed in PBS and lysed in 0.1 M KPO_4 (pH 7.8), 1.0% TRITON X-100, 1.0 mM DTT, 2 mM EDTA. Luciferase activities were measured as described by deWet et al. in *Mol. Cell. Biol.* 7:725 (1987).

TABLE 12

Compound	K_d (nM) @ RAR-γ[a]	EC_{50} (nM) @ RAR-γ[b]
ATRA	12	17
AGN 193109 (Compound 60)	6	na
AGN 193174 (Compound 34a)	52	na
AGN 193199	30	na
AGN 193385 (Compound 23)	25	na
AGN 193389 (Compound 25)	13	na
AGN 193840	40	na
AGN 193871 (Compound 50)	30	na

[a]Relative affinity (Kd) determined by competition of ^{3}H-ATRA binding to baculovirus expressed RAR-γ and application of the Cheng-Prussof equation.
[b]EC_{50} measured in CV-1 cells transiently cotransfected with ΔMTV-TREp-Luc and RS-RAR-γ. "na" denotes no activity.

As indicated by the results presented in part in FIG. 7 and in Table 12, with the exception of ATRA, all of the compounds listed in Table 12 were retinoid antagonists at RAR-γ.

The RAR-γ antagonists identified in Table 12 were next screened to determine which, if any, were also retinoid negative hormones. 4×10^4 CV-1 cells were transfected according to the calcium phosphate procedure described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al. eds. Cold Spring Harbor Lab Publ. 1989) using 0.5 μg ERE-tk-Luc reporter plasmid and 0.1 μg ER-RXR-α (Graupner et al. *Biochem. Biophys. Res. Comm.* 179:1554 (1991)) and 0.2 μg RAR-γ-VP-16 (Nagpal et al. *EMBO J.* 12:2349 (1993)) chimeric expression plasmids. After 18 hours, cells were rinsed with PBS and fed with DMEM (Gibco-BRL) containing 10% activated charcoal extracted FBS (Gemini Bio-Products). Cells were treated with 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M of each of the compounds listed in Table 12. Treatment with ethanol vehicle alone served as the negative control. After 18 hours, cells were rinsed in PBS and lysed in 0.1 M KPO_4 (pH 7.8), 1.0% TRITON X-100, 1.0 mM DTT, 2 mM EDTA. Luciferase activities were measured as previously by deWet et al. in *Mol Cell. Biol.* 7:725 (1987).

As shown in FIG. 8, the retinoid antagonists of Table 12 could be separated into two classes by virtue of their effect on the constitutive transcription activation function of the RAR-γ-VP-16 chimeric retinoid receptor. One group, which included AGN 193174, AGN 193199 and AGN 193840, did not repress RAR-γ-VP-16 activity even though they were ATRA antagonists. In contrast AGN 193109, AGN 193385, AGN 193389 and AGN 193871 exhibited a dose dependent repression of RAR-γ-VP-16 constitutive activity. Therefore, while the compounds of both groups were RAR-γ antagonists, only those of the second group exhibited negative hormone activity. This assay advantageously distinguished retinoid negative hormones from simple retinoid antagonists.

The foregoing experimental results proved that AGN 193109 met the criteria that define a negative hormone. More specifically, the results presented under Example 11 demonstrated that AGN 193109 had the capacity to exert

inhibitory activity at the RARs even in the absence of exogenously added retinoid ligands. As such, this compound possessed biological activities that did not depend upon blockade of the interaction between the RARs and agonists such as ATRA and AGN 191183. These findings led us to conclude that AGN 193109 stabilized interactions between RARs and NCPs. As diagrammed in FIG. 9, NCP/RAR/PCP interactions exist in an equilibrium state. An agonist serves to increase PCP interactions and decrease NCP interactions, while an inverse agonist or negative hormone stabilizes NCP and decreases PCP interactions. As indicated previously, our experimental results suggested that the intracellular availability of NCP for other receptors can be modulated by AGN 193109 administration. More specifically, we discovered that AGN 193109 can promote complexation of NCP with RARs, thereby reducing the intracellular reservoir of NCP available for interaction with transcription factors other than the RARs.

We next examined the effect of AGN 193109 on agonist-mediated inhibition of AP-1 dependent gene expression. In *Endocr. Rev.* 14:651 (1993), Pfhal disclosed that retinoid agonists can down-regulate gene expression by a mechanism that involved inhibition of AP-1 activity. We postulated that AGN 193109 could have had either of two effects when used in combination with a retinoid agonist in a model system designed to measure AP-1 activity. First, AGN 193109 could conceivably have antagonized the effect of the agonist, thereby relieving the agonist-dependent inhibition of AP-1 activity. Alternatively, AGN 193109 could have potentiated the agonist's activity, thereby exaggerating the agonist-dependent inhibition of AP-1 activity.

Example 13 describes the methods used to demonstrate that AGN 193109 potentiated the anti-AP-1 activity of a retinoid agonist. As disclosed below, the AGN 191183 retinoid agonist weakly inhibited AP-1 dependent gene expression. The combination of AGN 193109 and the retinoid agonist strongly inhibited AP-1 dependent gene expression. By itself, AGN 193109 had substantially no anti-AP-1 activity.

EXAMPLE 13

AGN 193109 Potentiates the Anti-AP-1 Activity of a Retinoid Agonist

HeLa cells were transfected with 1 μg of the Str-AP1-CAT reporter gene construct and 0.2 μg of plasmid pRS-hRARα, described by Giguere et al. in *Nature* 33:624 (1987), using LIPOFECTAMINE (Life Technologies, Inc.). Str-AP1-CAT was prepared by cloning a DNA fragment corresponding to positions −84 to +1 of the rat stromelysin-1 promoter (Matrisian et al., *Mol. Cell. Biol.* 6:1679 (1986)) between the HindIII-BamHI sites of pBLCAT3 (Luckow et al., *Nucl. Acids Res.* 15:5490 (1987)). This sequence of the stromelysin-1 promoter contains an AP1 motif as its sole enhancer element (Nicholson et al., *EMBO J.* 9:4443 (1990). The promoter sequence was prepared by annealing two synthetic oligonucleotides having sequences: 5'-AGAAGCTTATGGAAGCAATTATGAGTCAGTTTG-CGGGTGACTCTGCAAAT ACTGCCACT CTATAAAAGTTGGGCTCAGAAAGGTGGACCTCGA-GGATCCAG-3' (SEQ ID NO:2), and 5'-CTGGATCCTCG AGGTCCACCTTTCTGAGCCCAACTTT-TATAGAGTGGCAG TATTTGCA GAGTCACCCGCAAACTGACTCATAATTGCTTCCAT-AAGCTTCT-3' (SEQ ID NO:3). Procedures involving transfection, treatment with appropriate compounds and measurement of CAT activity were carried out as described by Nagpal et al. in *J. Biol. Chem.* 270:923 (1995), the disclosure of which is hereby incorporated by reference.

The results of these procedures indicated that AGN 193109 potentiated the anti-AP-1 activity of the retinoid agonist, AGN 191183. More specifically, in the concentration range of from 10^{-12} to 10^{-10} M, AGN 191183 did not inhibit the TPA-induced Str-AP1-CAT expression. Treatment with AGN 193109 in the concentration range of from 10^{-10} to 10^{-8} M did not substantially inhibit AP-1 mediated reporter activity. However, the results presented in FIG. 10 indicated that stimulation of the transfectants with the combination of AGN 193109 (10^{-8} M) and AGN 191183 in the concentration range of from 10^{-12} to 10−10 M substantially inhibited TPA-induced Str-AP1-CAT expression by an amount of from 12% to 21%. Therefore, AGN 193109 potentiated the anti-AP-1 activity of AGN 191183 under conditions where this retinoid agonist ordinarily did not inhibit AP-1 activity.

We reasoned that AGN 193109 potentiated the agonist-mediated repression of AP-1 activity by a mechanism that likely involved AGN 193109-dependent sequestration of NCPs onto RARs. RARs belong to a superfamily of nuclear receptors that also includes receptors for 1,25-dihydroxyvitamin D_3, glucocorticoid, thyroid hormone, estrogen and progesterone. It was a reasonable assumption that the ability to bind NCPs may be shared among different members of the nuclear receptor superfamily. This led us to speculate that AGN 193109 could potentiate the anti-AP-1 activity of one or more of the ligands that interact with this superfamily of nuclear receptors.

The results presented in the preceding Example clearly indicated that AGN 193109 potentiated the anti-AP-1 activity of a retinoid agonist. More specifically, AGN 193109 lowered the threshold dose at which the anti-AP-1 activity of AGN 191183 could be detected. Since AGN 193109 has substantially no anti-AP-1 activity by itself, its effect on nuclear receptor agonists was synergistic. We also found that the AGN 193109 negative hormone potentiated the anti-AP-1 activity of 1,25-dihydroxyvitamin D_3, the natural ligand for the vitamin D_3 receptor.

The observed synergy between AGN 193109 and AGN 191183 in the preceding Example necessarily implied that the anti-AP-1 activity of the retinoid agonist and the AGN 193109-mediated potentiation of that activity must result from different mechanisms. If the mechanisms of action of the two agents were identical, then it follows that the effectiveness of the combination of AGN 193109 and the agonist would have been additive. Instead, the combination was shown to be more effective than either agent alone, an effect that could not have been predicted in advance of this finding.

Significantly, the AGN 193109-mediated potentiation of the RAR agonist was performed using an approximately 100-fold molar excess of AGN 193109 over that of the retinoid agonist. Accordingly, the majority of RARs should have been bound by AGN 193109 leaving very few RARs available for agonist binding. In spite of this fact, the population of RARs that were not bound by AGN 193109 were able to bind retinoid agonist and vigorously stimulate an agonist-dependent response measurable as an inhibition of reporter gene expression. Thus, our data suggested possible heterogeneity of RARs that are induced by AGN 193109.

The negative hormone activity of AGN 193109, attributed to its ability to promote the interaction of RARs and NCPs, provided a basis for understanding the synergy between

AGN 193109 and retinoid agonists. Our results were fully consistent with a model in which AGN 193109 treatment of cells promoted binding of RARs and NCPs, thereby reducing the number of free NCP and free RAR within the cell. This results in the generation of two populations of RARs that are functionally distinct. The first population is represented by RARs associated with NCPs. Such AGN 193109/RAR/NCP complexes cannot be activated by retinoid agonists. The second population consists of RARs that are not bound by NCP, and that remain available for interaction with agonists. This latter population is designated "RAR*" to indicate free RARs in an environment substantially depleted of NCP.

The RAR*s have decreased probabilities of association with NCP through equilibrium binding and have an increased sensitivity to retinoid agonists measurable, for example, as anti-AP-1 activity. This is so because, while the intracellular reservoir of NCP is depleted by virtue of AGN 193109 administration, the reservoir of PCP has not been depleted. Accordingly, free RAR*s can bind a retinoid agonist and interact with PCP factors in an environment substantially depleted of NCP. The ability of AGN 193109 to increase the sensitivity of other nuclear receptors to their respective agonists can be attributed to the ability of these different nuclear receptors to interact with the same NCPs that interact with AGN 193109/RAR complexes. This model of AGN 193109-mediated modulation of NCP availability for nuclear receptor family members is schematically represented in FIG. 11.

This mechanistic model led us to predict that AGN 193109 could modulate the activities of nuclear receptor ligands other than retinoid agonists. As illustrated in the following Example, we confirmed that AGN 193109 potentiated the activity of 1,25-dihydroxyvitamin D_3 in an in vitro transactivation assay.

Example 14 describes the methods used to demonstrate that AGN 193109 enhanced the activity of 1,25-dihydroxyvitamin D_3 in a transactivation assay.

EXAMPLE 14

AGN 193109 Potentiates 1,25-Dihydroxyvitamin D, Activity

Hela cells were transfected using the cationic liposome-mediated transfection procedure described by Felgner et al. in *Proc. Natl. Acad. Sci. USA* 84:7413 (1987). 5×10^4 cells were plated in 12-well multiwell plates and grown in DMEM supplemented with 10% FBS. Cells were cotransfected in serum-free medium using 2 μg/well of LIPOFECTAMINE reagent (Life Technologies, Inc.) with 0.7 μg of the reporter plasmid MTV-VDRE-Luc, containing two copies of the 1,25-dihydroxyvitamin D_3 response element 5'-GTACAAGGTTCACGAGGTTCACGTCTTA-3' (SEQ ID NO:4) from the mouse osteopontin gene (Ferrara et al. *J. Biol. Chem.* 269:2971 (1994)) ligated into the reporter plasmid AMTV-Luc (Heyman et al. in *Cell* 68:397 (1992)), and 0.3 μg of the plasmid pGEM3Z (Pharmacia, Inc.) as carrier DNA to bring the final concentration of DNA to 1.0 μg per well. After six hours of transfection, cells were fed with growth medium containing charcoal extracted FBS at a final concentration of 10%. Eighteen hours after transfection cells were treated with vehicle alone (ethanol) or AGN 193109 in ethanol at a final concentration of either 10^{-8} or 10^{-7} M. Six hours later 1,25-dihydroxyvitamin D_3 was added in ethanol to a final concentration of from 10^{-10} to 10^{-7} M. Cells were lysed and harvested eighteen hours following 1,25-dihydroxyvitamin D_3 treatment. Luciferase activity was measured as described above. This experimental system allowed a convenient method of monitoring and quantitating 1,25-dihydroxyvitamin D_3-dependent gene expression.

The results presented in FIG. 12 indicated that, when compared with the result obtained using 1,25-dihydroxyvitamin D_3 alone, AGN 193109 coadministered with 1,25-dihydroxyvitamin D_3 shifted the dose response curve to the left. This confirmed that AGN 193109 potentiated the effectiveness of 1,25-dihydroxyvitamin D_3 in the in vitro transactivation assay. More specifically, FIG. 12 graphically illustrates that an AGN 193109 concentration as low as 10–100 nM rendered the 1,25-dihydroxyvitamin D_3 approximately 10 fold more active. While a 1,25-dihydroxyvitamin D_3 concentration of 10^{-8} M was required to produce a luciferase expression of approximately 2,000 rlu, only one-tenth as much 1,25-dihydroxyvitamin D_3 was required to produce the same luciferase output when the vitamin was coadministered with AGN 193109 at a concentration of 10^{-8}–10^{-7} M. Although not shown on the graph in FIG. 12, substantially identical results were obtained using AGN 193109 concentrations of 10^{-9} M and 10^{-8} M. Thus, coadministration with AGN 193109 substantially reduced the amount of 1,25-dihydroxyvitamin D_3 that was required to produce a similar effect in the absence of the negative hormone.

Interestingly, when the above procedure was repeated with cotransfection of a vitamin D receptor (VDR) expression plasmid, there was a coincident decrease in the ability of AGN 193109 to potentiate the activity of 1,25-dihydroxyvitamin D_3. We interpreted this result as indicating that over-expression of VDRs could affect the ability of AGN 193109 to potentiate the activity of 1,25-dihydroxyvitamin D_3. Thus, the intracellular concentration of a ligand receptor, which may differ in a tissue-specific fashion, can influence the ability of AGN 193109 to potentiate the activity of a ligand that binds the receptor. This was again consistent with a model in which titratable NCPs contributed to the regulation of the Vitamin D_3 response, and supported the model set forth above.

As illustrated in the following Example, we also confirmed that AGN 193109 potentiated the anti-AP-1 activity of 1,25-dihydroxyvitamin D_3. Our model for the activity of AGN 193109 action explains this observation by invoking that NCPs avidly associate with RARs in the presence of this drug. Endogenous vitamin D receptors present in HeLa cells likely were rendered more sensitive to the 1,25-dihydroxyvitamin D_3 ligand, with the consequence of exaggerating the ability of this ligand to inhibit expression from the Str-AP1-CAT reporter.

Example 15 describes the methods used to demonstrate that AGN 193109 potentiated the anti-AP-1 activity of 1,25-dihydroxyvitamin D_3.

EXAMPLE 15

AGN 193109 Potentiates the Anti-AP-1 Activity of 1,25-Dihydroxyvitamin D_3

HeLa cells were transfected with 1 μg of Str-AP1-CAT using LIPOFECTAMINE according to the method described by Nagpal et al. in *J. Biol. Chem.* 270:923 (1995). Transfected cells were treated with AGN 193109 alone (10^{-9} to 10^{-7} M), 1,25-dihydroxyvitamin D_3 alone (10^{-12} to 10^{-7} M) or 1,25-dihydroxyvitamin D_3 (10^{-12} to 10^{-7} M) in the presence of 10^{-8} M AGN 193109.

The results of these procedures indicated that AGN 193109 potentiated the ability of 1,25-dihydroxyvitamin D_3 to inhibit TPA-induced AP-1 activity. When used alone in the concentration range of from 10^{-9} to 10^{-7} M, AGN 193109 had no detectable anti-AP-1 activity. The results presented in FIG. **13** indicated that 1,25-dihydroxyvitamin D_3 repressed TPA-stimulated activity only in the 10^{-8} and 10^{-7} M concentration range. Analysis of 1,25-dihydroxyvitamin D_3 mediated repression of TPA stimulated CAT activity in the presence of 10^{-8} M AGN 193109 indicated that anti-AP-1 activity was detectable at 10^{-10} and 10^{-9} M 1,25-dihydroxyvitamin D_3 and an increase in activity at 10^{-8} and 10^{-7} M doses compared to 1,25-dihydroxyvitamin D_3 treatment alone. This AGN 193109 dependent modulation of 1,25-dihydroxyvitamin D_3 mediated anti-AP-1 activity was consistent with our model in which NCP sequestration to RARs made the NCP unavailable for interaction with other nuclear receptor family members. Accordingly, the receptors were rendered more sensitive to the 1,25-dihydroxyvitamin D_3 treatment.

The mechanisms underlying RAR mediated transactivation and anti-AP-1 activity are likely different. This conclusion was based on our observation that high doses of AGN 193109 completely inhibited transactivation without substantially inhibiting anti-API activity. We therefore wished to gain additional evidence to support our model for RAR* formation mediated by AGN 193109 treatment. To accomplish this, we investigated whether AGN 193109 could potentiate the activity of the RAR specific agonist AGN 191183 in an in vitro transactivation assay.

Example 16 describes the methods used to demonstrate that AGN 193109 potentiated the activity of the RAR specific agonist, AGN 191183. The results of this procedure indicated that, under particular circumstances, AGN 193109 enhanced the potency of the RAR specific retinoid, and provided strong evidence that AGN 193109 promoted RAR* formation.

EXAMPLE 16

Potentiation of Retinoid Effectiveness by AGN 193109 Coadministration

Hela cells were transfected using the cationic liposome-mediated transfection procedure described by Felgner et al. in *Proc. Natl. Acad. Sci. USA* 84:7413 (1987). 5×10^4 cells were plated in 12 well multiwell plates and grown in DMEM supplemented with 10% FBS. Cells were cotransfected in serum free medium using LIPOFECTAMINE reagent (2 μg/well, Life Technologies, Inc.) with 0.7 μg of the reporter plasmid MTV-TREp-Luc, containing two copies of the TREpal response element 5'-TCAGGTCATGACCTGA-3' (SEQ ID NO:5) inserted into the reporter plasmid ΔMTV-Luc (Heyman et al. in *Cell* 68:397 (1992)), and 0.1 μg of the RAR-γ expression plasmid pRShRAR-γ (Ishikawa et al. *Mol. Endocrinol.* 4:837 (1990)).

After six hours of transfection, cells were fed with growth medium containing charcoal extracted FBS at a final concentration of 10%. Eighteen hours after transfection, cells were treated with vehicle alone (ethanol) or AGN 193109, in ethanol at a final concentration of from 10^{-11} to 10^{-8} M. Six hours later, AGN 191183 was added in ethanol to a final concentration of either 0, 10^{-10} or 10^{-9} M. Cells were harvested after eighteen hours of AGN 191183 treatment and luciferase activity was measured as described above.

Preliminary experiments indicated that 10^9 M AGN 193109 was relatively ineffective at inhibiting the response to of 10^{-9} M AGN 191183 in HeLa cells. This contrasted with the ability of 10^{-9} M AGN 193109 to inhibit 10^{-8} M ATRA in CV-1 cells (FIG. **2**).

The results presented in FIG. **14** supported the prediction that AGN 193109 stimulated the formation of RAR*. Consistent with our characterization of the antagonist and negative hormone activities of AGN 193109, treatment with AGN 193109 resulted in a biphasic dose response curve. The lowest doses of AGN 193109 (10^{-11} and 10^{-10} M) resulted in a stimulation of luciferase activity over that of AGN 191183 alone. This effect suggests that RAR*s are generated by AGN 193109. Curiously, this was also seen for AGN 193109 treatment alone, suggesting that RAR*'s can respond to an endogenous ligand. AGN 191183 is a synthetic retinoid agonist and, like ATRA, activates transcription through the RARs. Substitution of AGN 191183 for ATRA in Example 7 would give qualitatively similar results (i.e., AGN 193109 would antagonize the effect of 10 nM AGN 191183). Example 16 illustrates that, while AGN 193109 can function as an antagonist of RAR agonists, dosing conditions could easily be identified wherein AGN 193109 coadministration potentiated activation mediated by an RAR agonist. It is important to note that the doses of the compounds used in Example 16 are substantially lower than the doses employed in the procedure described under Example 7. We proposed that AGN 193109 treatment could lead to RAR heterogeneity RARs versus RAR*s. The apparent heterogeneity (i.e., ability to potentiate) appears to have different windows in transactivation versus AP-1 repression. The reason that the curves are biphasic is because, with increasing amounts of AGN 193109, there is proportionately less RAR available to bind the agonist. This doesn't appear to be the case for AP-1 repression and we are left to speculate that this difference must reflect two distinct mechanisms for transactivation and AP-1 repression by the same receptor species.

Clinical results have confirmed that some retinoids are useful for inhibiting the growth of premalignant and malignant cervical lesions. Exemplary studies supporting this conclusion have been published by Graham et al. in *West. J. Med.* 145: 192 (1986), by Lippman et al. in *J. Natl. Cancer Inst.* 84:241 (1992), and by Weiner et al. in *Invest. New Drugs* 4:241 (1986)).

Similar conclusions are supported by the results of in vitro studies that used cultured cells to quantitate the antiproliferative effects of various retinoids. More specifically, Agarwal et al. in *Cancer Res.* 51:3982 (1991) employed the ECE16-1 cell line to model the early stages of cervical dysplasia and demonstrated that retinoic acid could inhibit epidermal growth factor (EGF) dependent cellular proliferation.

Example 17 describes the methods used to demonstrate that AGN 193109 can antagonize the activity of the AGN 191183 retinoid agonist which inhibited proliferation of the ECE 16-1 cell line.

EXAMPLE 17

AGN 193109 Antagonizes the Antiproliferative Effect of Retinoids in ECE16-1 Cells

ECE16-1 cells were seeded at a density of 1×10^4 cells per cm^2 in complete medium containing DMEM:F12 (3:1), nonessential amino acids, 5% FBS, 5 μg/ml transferrin, 2 nM of 3,3',5 triiodothyronine (thyroid hormone or "T_3"), 0.1 nM cholera toxin, 2 mM L-glutamine, 1.8×10-4 M adenine and 10 ng/ml EGF. Cells were allowed to attach to plates

overnight and then shifted to defined medium containing DMEM:F12 (3:1), 2 mM L-glutamine, nonessential amino acids, 0.1% bovine serum albumin, 1.8×10–4 M adenine, 5 μg/ml transferrin, 2 nM T_3, 50 μg/ml ascorbic acid, 100 ug/ml streptomycin, 100 units/ml penicillin and 50 μg/ml gentamicin. Defined medium (DM) was supplemented with 10 ng/ml EGF. EGF treated cells received 10 nM of the AGN 191183 retinoid agonist in combination with either 0, 0.1, 1.0, 10, 100 or 1000 nM AGN 193109 or 1000 nM AGN 193109 alone. After three days of treatment, cells were harvested as described by Hembree et al. in *Cancer Res.* 54:3160 (1994) and cell numbers determined using a COULTER counter.

The results presented in FIG. **15** demonstrated that ECE16-1 cells proliferated in response to EGF but not in defined medium alone. This corfirmed the findings published by Andreatta-van Leyen et al. in *J. Cell. Physio.* 160:265 (1994), and by Hembree et al. in *Cancer Res.* 54:3160 (1994). Addition of 10 nM AGN 191183 and 0 nM AGN 193109 completely inhibited EGF mediated proliferation. Thus, AGN 191183 was a potent antiproliferative retinoid. Increasing the AGN 193109 concentration from 0 nM to 10 nM antagonized the AGN 191183 mediated growth inhibition by approximately 50%. A ten-fold molar excess of AGN 193109 completely reversed the antiproliferative effect of AGN 191183. Treatment of cells with 1000 nM AGN 193109 alone had no effect on the EGF mediated proliferation increase. These results proved that AGN 193109 antagonized the antiproliferative effect of a retinoid but had substantially no antiproliferative activity of its own when used to treat cells representing cervical epithelium that is sensitive to growth inhibition by retinoids such as AGN 191183. Notably, there was no evidence that AGN 193109 potentiated the antiproliferative activity of the AGN 191183 agonist using the ECE 16- 1 model system.

In contrast to the model system represented by the ECE16-1 cell line, there are other examples where cellular proliferation associated with cervical dysplasia cannot be inhibited by retinoid agonists. For example, Agarwal et al. in *Cancer Res.* 54:2108 (1994) described the use of CaSki cells as a model for cervical tumors that are unresponsive to retinoid therapy. As disclosed below, rather than inhibiting cell proliferation, retinoid treatment had substantially no effect on the growth rate of CaSki cells. The following Example addressed the effect of the AGN 193109 negative hormone on the proliferation rates of this cell line. The results unexpectedly proved that AGN 193109 can inhibit the proliferation of cervical tumor cells that are unresponsive to the antiproliferative effects of retinoid agonists.

Example 18 describes the methods used to demonstrate that AGN 193109 inhibited the growth of a cervical tumor cell line that did not respond to the antiproliferative effects of other retinoids such as AGN 191183. Significantly, AGN 193109 displayed antiproliferative activity in the absence of added retinoid

EXAMPLE 18

AGN 193109 Inhibits the Proliferation Rate of CaSki Cervical Carcinoma-Derived Cell Line

We tested the effect of EGF on CaSki cell proliferation, either alone or in combination with the AGN 191183 retinoid agonist and/or the AGN 193109 negative hormone at a concentration of 10^{-6} M. Cell proliferation assays were performed as described above for studies involving ECE16-1 cells. EGF was added to the retinoid treated cultures to give a final concentration of 20 ng/ml. Cells were treated with AGN 191183 (10^{-10} to 10^{-6} M) in the presence or absence of 10^{-6} M AGN 193109 for a total of three days. The media was replaced with fresh media and each of the two retinoid compounds, as appropriate, every day. Cell numbers were determined using a COULTER counter as described above.

The results presented in FIG. **16** indicated that CaSki cells were substantially refractory to the effects of a retinoid agonist and that AGN 193109 exhibited antiproliferative activity in the absence of added retinoid. The presence of EGF in the culture media stimulated CaSki cell growth. This conclusion was based on comparison of the stripped bar representing no AGN 191183 and the open bar representing defined growth media ("DM") alone. AGN 191183 treatment had no antiproliferative activity on the CaSki tumor cell line. We discounted any slight increase in the cellular proliferation rate associated with the retinoid agonist, because a ten thousand fold increase in the retinoid agonist concentration was associated with only roughly a 20% increase in the proliferation rate. Thus, the AGN 191183 agonist had substantially no effect on the proliferation rate of CaSki cells.

The results presented in FIG. **16** also indicated that AGN 193109 inhibited proliferation of the CaSki cervical epithelial cell line. This conclusion was based on comparison of the measurements appearing as the "0" AGN 191183 black bar and the "0" AGN 191183 stripped bar. Thus, AGN 193109 was capable of stimulating a biological response in the absence of added retinoid agonist when used to treat cervical tumor cells that were not growth inhibited by retinoid agonists such as AGN 191183.

Our discovery that the AGN 193109 negative hormone could inhibit cellular proliferation was consistent with a model in which unliganded RAR mediated the expression of genes that were required for proliferation. While an RAR agonist such as AGN 191183 had substantially no effect, or perhaps promoted cellular proliferation slightly, AGN 193109 had an antiproliferative effect. The AGN 193109 negative hormone likely bound RARs thereby promoting NCP association and causing the RARs to adopt an inactive conformation. According to our model, this repressed gene activity that was positively regulated by unliganded RARs. This ability of AGN 193109 to down-regulate the activity of unliganded RARs likely resulted from its ability to promote the association of RARs and NCPs.

Those having ordinary skill in the art will appreciate that some retinoid agonists are useful for controlling the undesirable consequences of cell growth that follows retinal detachment. After retinal detachment the retinal pigment epithelium (RPE) dedifferentiates, proliferates and migrates into the subretinal space. This process can negatively impact the success of surgical procedures aimed at retinal reattachment. Campochiaro et al. in *Invest. Opthal & Vis. Sci.* 32:65 (1991) have demonstrated that RAR agonists such as ATRA exhibited an antiproliferative effect on the growth of primary human RPE cultures. Retinoid agonists have also been shown to decrease the incidence of retinal detachment following retinal reattachment surgery (Fekrat et al. *Opthamology* 102:412 (1994)). As disclosed in the following Example, we analyzed the ability of the AGN 193109 negative hormone to suppress growth in primary human RPE cultures.

Example 19 describes the methods used to demonstrate that AGN 193109 potentiated the antiproliferative effect of a retinoid antagonist in a primary culture of human retinal pigment epithelium.

EXAMPLE 19

AGN 193109 Potentiates the Antigroliferative Activity of ATRA

Primary cultures of human retinal pigment epithelium (RPE) were established according to the method described by Campochiaro et al. in *Invest. Opthal & Vis. Sci.* 32:65 (1991). 5×10^4 cells were plated in 16-mm wells of 24-well multiwell plates in DMEM (Gibco) containing 5% FBS. Cells were mock treated with ethanol vehicle alone, ATRA (10^{-10} to 10^{-6} M) in ethanol, AGN 193109 (10^{-10} to 10^{-6} M) in ethanol, or ATRA (10^{-10} to 10^{-6} M) and 10^{-6} M AGN 193109. Cells were fed with fresh media containing the appropriate concentrations of these compounds every two days for a total of five days of treatment. Cells were removed from the plates by gentle digestion with trypsin and the number of cells was counted with an electronic cell counter.

The results presented in FIG. **17** indicated that AGN 193109 dramatically potentiated the antiproliferative activity of ATRA on RPE cells. Treatment of primary RPE cells with ATRA led to a dose dependent decrease in RPE cell proliferation with an approximately 40% decrease at 10^{-6} M ATRA relative to control cultures. AGN 193109 treatment did not substantially alter the growth rate of the RPE cells at any concentration tested in the procedure. Unexpectedly, the combination of ATRA (10^{-11} to 10^{-6} M) and 10^{-6} M AGN 193109 had a stronger antiproliferative activity than ATRA alone. Thus, AGN 193109 cotreatment potentiated the antiproliferative effect of ATRA. More specifically, the results shown in the Figure indicated that the antiproliferative effect of 10^{-8} M ATRA was obtainable using only 10^{-10} M ATRA in combination with 10^{-7} M AGN 193109. Thus, the AGN 193109 negative hormone advantageously enhanced the antiproliferative activity of ATRA by approximately 100 fold.

In an independent experiment, comparison of the antiproliferative effect of ATRA (10^{-11} to 10^{-6} M) with that of ATRA and 10^{-6} M AGN 193109 again demonstrated the apparent increase in sensitivity of primary RPE cells to ATRA in the presence of AGN 193109. In this system, AGN 193109 neither functioned as a retinoid antagonist nor exhibited an antiproliferative effect when used alone. However, AGN 193109 coadministration potentiated the antiproliferative activity of the retinoid agonist.

AGN 193109 was tested for its ability to potentiate the anti-proliferative effect of 13-cis retinoic acid (13-cis RA) in primary RPE cultures using conditions and techniques to measure RPE cell proliferation described above. Notably, 13-cis RA is clinically significant. More particularly, 13-cis RA is useful in the treatment of several disease states, including acne (Peck et al. *N. Engl. J. Med.* 300:329 (1977); Jones et al. *Br. J Dermatol.* 108:333 (1980)), and squamous cell carcinoma of the skin and cervix in combination treatment with interferon 2α (Lippman et al. *J. Natl. Cancer Inst.* 84:241 (1992); Moore et al. *Seminars in Hematology* 31:31 (1994)).

The results presented in FIG. **18** indicated that both 13-cis RA (10^{-12} to 10^{-6}M) and ATRA (10^{-12} to 10^{6}M) effectively inhibited RPE cell growth. Notably, the 13-cis isomer was approximately two orders of magnitude less effective in this assay when compared with ATRA. Similar to the results obtained using coadministration of AGN 193109 and ATRA (above), coadministration of AGN 193109 (either 10^{-8} or 10^{-6}M) with 13-cis RA (10^{-12} to 10^{-6}M) dramatically increased the potency of 13-cis RA in mediating repression of RPE cell proliferation. In contrast to treatment with 13-cis RA alone, coadministration of AGN 193109 enhanced the potency of 13-cis RA. Thus, AGN 193109 potentiated the antiproliferative activity of 13-cis RA.

We next tested the ability of AGN 193109 to potentiate the activities of other nuclear receptor hormones in primary RPE cell cultures. Dexamethasone, a synthetic glucocorticoid receptor agonist, is one member of a class of compounds that have been used clinically for their potent anti-inflammatory and immunosuppressive properties. Thyroid hormone (T3; 3,3',5'-Triiodothyronine) is a natural thyroid hormone receptor agonist used primarily for hormone replacement therapy in the treatment of hypothyroidism. Methods used in these experiments were identical to those described above for procedures employing ATRA and 13-cis RA.

The results of these procedures indicated that coadministration of AGN 193109 and the nuclear receptor agonists potentiated the antiproliferative activities of the nuclear receptor agonists. More specifically, the results presented in FIG. **19** showed that single-agent treatment of RPE cells with either dexamethasone (10^{-11} to 10^{-6}M) or ATRA (10^{-12} to 10^{-6}M) was substantially unable to inhibit RPE cell proliferation. However, treatment of RPE cells with dexamethasone (10^{-11} to 10^{-6}M) and either 10^{-8} or 10^{-6}M AGN 193109 repressed RPE cell proliferation to an extent that approximated the inhibition caused by treatment with ATRA. Similarly, the results presented in FIG. **20** indicated that AGN 193109 potentiated the antiproliferative activity of thyroid hormone. Similar to the results obtained using dexamethasone, the proliferation of RPE cells was refractory to single-agent treatment with thyroid hormone (10^{-11} to 10^{-6}M). However, co-treatment of RPE cells with thyroid hormone (10^{-11} to 10^{-6}M) and AGN 193109 (either 10^{-8} or 10^{-6}M) inhibited RPE cell proliferation in a thyroid hormone dependent manner. We concluded that AGN 193109 rendered primary RPE cultures sensitive to the antiproliferative effects of these nuclear receptor agonists. The mechanism by which AGN 193109 mediated these effects likely involved modulation of NCP/RAR interactions.

We additionally examined the effect of AGN 193109 on the expression of marker genes in other experimental systems that were sensitive to retinoid agonists. Both the MRP8 and stromelysin genes are known to be inhibited by retinoid agonists in a variety of biological systems. For example, Wilkinson et al. in *J. Cell Sci.* 91:221 (1988) and Madsen et al. in *J. Invest. Dermatol.* 99:299 (1992) have disclosed that MRP8 gene expression was elevated in psoriasis. Conversely, MRP8 gene expression was repressed by the retinoid agonist AGN 190168 in human psoriatic skin (Nagpal et al., submitted 1995), in human keratinocyte raft cultures (Chandraratna et al. *J Invest. Dermatol.* 102:625 (1994)) and in cultured human newborn foreskin keratinocytes (Thacher et al. *J. Invest. Dermatol.* 104:594 (1995)). Nagpal et al. in *J. Biol. Chem.* 270:923 (1995) have disclosed that stromelysin mRNA levels were repressed by retinoid agonists such as AGN 190168 in cultured human newborn foreskin keratinocytes. We analyzed the regulated expression of these genes following treatment of cultured human newborn foreskin keratinocytes with either the AGN 191183 retinoid agonist or AGN 193109.

Example 20 describes the methods used to demonstrate that AGN 193109 inhibited MRP-8 expression in cultured keratinocytes.

EXAMPLE 20

AGN 193109 Inhibits MRP-8 Expression in Keratinocytes

Primary foreskin keratinocytes were isolated according to the procedure described by Nagpal et al. in *J. Biol. Chem.*

270:923 (1995) and cultured in keratinocyte growth medium (KGM) that was purchased from Clonetics. After 3 days of treatment with AGN 191183 (10^{-7} M) or AGN 193109 (10^{-6} M), total cellular RNA was isolated from treated and control keratinocytes according to standard methods. The mRNA was reverse transcribed into cDNA which then served as the template in a PCR amplification protocol using primers specific for either the glyceraldehyde phosphate dehydrogenase (GAPDH) housekeeping gene or MRP-8. The GAPDH primers had the sequences 5'-CCACCCATGGCAAATTCCATGGCA-3' (SEQ ID NO:6) and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (SEQ ID NO:7). The MRP-8 primers had the sequences 5'-ACGCGTCCGGAAGACCTGGT-3' (SEQ ID NO:8) and 5'-ATTCTGCAGGTACATGTCCA-3' (SEQ ID NO:9). An aliquot from the MRP-8 amplification reaction (10 μl) was removed after every cycle of PCR amplification starting from 12 cycles and ending at 21 cycles. Similarly, an aliquot of the GAPDH amplification reaction was removed after every PCR cycle starting at 15 cycles and ending at 24 cycles. The samples were electrophoresed on 2% agarose gels and the separated amplification products detected by ethidium bromide staining. The staining intensity of the amplification products served as a quantitative measure of the amount of starting mRNA specific for the given primer set.

The results of this procedure indicated that both AGN 191183 and AGN 193109 independently inhibited MRP-8 expression in keratinocytes. The intensity of the stained GAPDH amplification product was substantially equivalent in the lanes of the gel representing starting material isolated from control, AGN 191183, and AGN 193109 treated keratinocytes. Weak bands representing the GAPDH amplification product were first detectable in lanes corresponding to samples removed after 18 cycles of PCR amplification. The equivalent staining intensities among the various lanes of the gel indicated that equivalent masses of starting material were used for all samples. Accordingly, differences in the intensities of stained bands representing MRP-8 amplification products were indicative of differences in MRP-8 mRNA expression among the various starting samples. As expected, the MRP-8 amplified signal was inhibited in AGN 191183 (10^{-7} M) treated cultures relative to an untreated control. AGN 193109 (10^{-6} M) treatment of cultured keratinocytes also repressed MRP8 expression as judged by lower intensity of stained amplification product.

As illustrated in the following Example, AGN 193109 also inhibited expression of a second marker gene in keratinocytes. Nagpal et al. in *J. Biol. Chem.* 270:923 (1995) disclosed that stromelysin mRNA expression was down-regulated by RAR specific agonists in cultured newborn human foreskin keratinocytes. Nicholson et al. (*EMBO J.* 9:4443 (1990)) disclosed that an AP-1 promoter element played a role in the retinoid-dependent negative regulation of the stromelysin-1 gene. Thus, it was of interest to determine whether AGN 193109 could alter the expression of this gene.

Example 21 describes the methods used to demonstrate that AGN 193109 inhibited stromelysin-1 gene expression in the absence of an exogenously added retinoid agonist.

EXAMPLE 21

AGN 193109 Inhibits Stromelysin-1 Expression in Cultured Keratinocytes

Primary foreskin keratinocytes were either mock treated or treated for 24 hours with the RAR agonist AGN 191183 (10^{-7} M), or AGN 193109 (10^{-6} M). Total RNA prepared from mock-treated and retinoid-treated keratinocytes was reverse transcribed and the resulting cDNA was PCR amplified using β-actin or stromelysin-1 oligo primers exactly as described by Nagpal et al. in *J. Biol. Chem.* 270:923 (1995)), the disclosure of which has been incorporated by reference. A sample (10 μl) from the PCR amplification reaction was removed after every three cycles starting from 18 cycles of PCR amplification. The sample was electrophoresed on a 2% agarose gel and detected after ethidium bromide staining.

Results of these procedures indicated that AGN 193109 inhibited stromelysin-1 gene expression in the absence of an exogenously added retinoid agonist. More specifically, ethidium-stained bands representing β-actin amplification products were easily detectable the agarose gels after 18 cycles of PCR. While all band intensities increased with additional cycles of the amplification reaction, stained bands were somewhat less intense in samples representing AGN 191183 treated cells. This indicated that a slightly lesser amount of RNA must have been present in the starting samples corresponding to cells treated with AGN 191183. The results also indicated that stromelysin-1 mRNA was detectable in mock-treated keratinocytes starting at 33 cycles of PCR amplification. As expected, stromelysin-1 MRNA expression was inhibited after AGN 191183 (10^{-7} M) treatment as judged by the weaker band intensity on when compared with samples derived from mock-treated samples. When normalized to the intensities of the β-actin amplification products, and consistent with the results obtained in measurements of MRP-8 expression, AGN 193109 (10^{-6} M) treatment of keratinocytes resulted in down-regulation of stromelysin-1 MRNA levels. Indeed, the down-regulation stimulated by AGN 193109 treatment was indistinguishable from the down-regulation caused by treatment of keratinocytes with the RAR agonist AGN 191183.

As disclosed herein, AGN 193109 can have any of three possible effects with respect to modulating the activity of a coadministered steroid superfamily agonist. First, AGN 193109 may have no effect. Second, AGN 193109 may antagonize the effect of the agonist, thereby leading to a decrease in the activity of the agonist. Finally, AGN 193109 may potentiate the activity of the agonist, thereby leading to a stimulation of the measured effect produced by the agonist.

Compounds having activities that can be modulated by AGN 193109 include retinoid receptor agonists and agonists which bind to other members of the steroid receptor superfamily. This latter category of agonists includes vitamin D receptor agonists, glucocorticoid receptor agonists and thyroid hormone receptor agonists. Peroxisome proliferator-activated receptors, estrogen receptor and orphan receptors having presently unknown ligands may also be potentiated by AGN 193109. In the case where the steroid superfamily agonist is an RAR agonist, AGN 193109 may either antagonize or potentiate the activity of that agonist. In the case where the agonist used in combination with AGN 193109 is a compound that can bind to a nuclear receptor other than an RAR, coadministration of AGN 193109 will either have no effect or will sensitize of the system to the agonist so that the activity of the agonist is potentiated.

A generalized exemplary procedure for determining which of the three possible activities AGN 193109 will have in a particular system follows. This description illustrates each of the possible outcomes for AGN 193109 coadministration with a steroid receptor superfamily agonist. Biological systems useful for assessing the ability of AGN 193109 to modulate the activity of a nuclear receptor agonist

include but are not limited to: established tissue culture cell lines, virally transformed cell lines, ex-vivo primary culture cells and in vivo studies utilizing living organisms. Measurement of the biological effect of AGN 193109 in such systems could include determination of any of a variety of biological endpoints. These endpoints include: analysis of cellular proliferation, analysis of programmed cell death (apoptosis), analysis of the differentiation state of cells via gene expression assays, analysis of the ability of cells to form tumors in nude mice and analysis of gene expression after transient or stable introduction of reporter gene constructs.

For illustrative purposes, an MRNA species designated as mRNA "X" is expressed from gene "X" in primary cultured "Y" cells isolated from the organ "Z." Under standard culture conditions, where several "Y" cell genetic markers are maintained, including expression of gene "X", addition of a retinoid agonist leads to a decrease in the abundance of "X" mRNA. Analysis of gene X expression can be assessed via isolation of cellular mRNA and measurement of the abundance of X mRNA levels via polymerase chain reaction, ribonuclease protection or RNA blotting procedures such as Northern analyses. After isolation from organ Z, primary Y cells are cultured in an appropriate growth medium. The primary cultures are then plated into tissue culture plates for expansion of the cell population. This step facilitates separation of the cells into four sample groups so that various doses of the retinoid agonist and AGN 193109 can be delivered. The first group will be a control, receiving vehicle only. The second group will receive the RAR agonist, retinoic acid, delivered in ethanol, in amounts sufficient to provide final concentrations in the range of from 10^{-11} to 10^{-6} M. The lowest dose may need to be empirically determined depending on the sensitivity of the system. Such determinations fall within the scope of routine experimentation for one having ordinary skill in the art. The third group will receive both the nuclear receptor agonist at the same doses used for treating the cells of group 2, and a constant dose of AGN 193109. The dose of AGN 193109 used for treating the cells of group 3 will also need to be determined empirically, but should approximate the affinity constant (Kd) of AGN 193109 for the RAR subtypes (i.e., at least 10^{-8} M). The fourth group will receive AGN 193109 at doses minimally including that used for agonist coadministration in group 3. An alternative to this dosing regimen would substitute AGN 193109 for the retinoid agonist described in the foregoing example, as specified in group 2, and a constant dose of retinoid agonist in place of AGN 193109, as specified in groups 3 and 4. After a suitable incubation period, cells should be harvested in a manner suitable for determination of the biological endpoint being measured as an indicator of agonist activity.

For example, analysis of the effect of AGN 193109 on retinoic acid dependent regulation of gene expression would involve comparison of the abundance of the MRNA species X in the mRNA pool harvested from cells treated according to each of the four protocols described above. RNA derived from control cells will serve to determine the baseline expression of X mRNA and will represent a condition corresponding to no repression. Comparison of this level with that measured in the mRNA pool derived from cells treated with retinoic acid will allow for determination of the effect of this agonist on gene expression. Quantitated levels of the repression of specific mRNAs resulting from retinoic acid treatment can then be compared with mRNA abundances from cells treated in parallel with either AGN 193109 alone or AGN 193109 in combination with retinoic acid.

While this generalized example illustrates an analysis of the effect of coadministered AGN 193109 on the expression of a gene repressed by a retinoid agonist, the example could alternatively have described analysis of the effect of coadministered AGN 193109 on a gene that was induced by a retinoid agonist. The critical feature for determining whether AGN 193109 will behave as an agonist, as a negative hormone or have no effect in a particular system will involve quantitative comparison of the magnitude of the effect in the presence and absence of AGN 193109.

An example in which AGN 193109 potentiated the activity of a coadministered agonist would be a case in which AGN 193109 cotreatment with retinoic acid resulted in a level of X mRNA expression that is further repressed relative to the level measured in cells treated with retinoic acid alone. More specifically, comparison of the dose response curve of the biological effect (i.e., repression of X mRNA abundance) plotted on the Y-axis versus the dose of the agonist (logarithmic scale) on the X-axis would allow comparison of agonist-mediated repression of X mRNA abundance in the presence and absence of AGN 193109 cotreatment. The ability of AGN 193109 to sensitize the biological response to the agonist, thereby potentiating the activity of the agonist, will be indicated by a leftward shift in the dose response curve. More specifically, in the presence of AGN 193109 less agonist would be required to obtain the same biological effect obtainable using the agonist alone.

An example of AGN 193109 mediating antagonism of a coadministered agonist would be a case in which AGN 193109 cotreatment with retinoic acid resulted in a level of X mRNA expression that is less repressed compared to that measured in cells treated with retinoic acid alone. Comparison of dose response curves of X mRNA repression versus log dose of agonist in the presence and absence of AGN 193109 will demonstrate a shift to the right in the dose response curve. More specifically, in the presence of AGN 193109, more agonist will be necessary to obtain the same biological effect obtainable with single agent treatment with the agonist alone.

The above examples wherein AGN 193109 mediates either antagonism or potentiation describe experimental outcomes for coadministration of AGN 193109 with a retinoid agonist. If, however, the agonist coadministered with AGN 193109 is an agonist capable of binding and activating a member of the steroid receptor superfamily other than an RAR, then instead of antagonizing the agonist, it becomes possible that AGN 193109 would have no effect on the activity of the agonist. If AGN 193109 cotreatment with such an agonist results in a level of mRNA expression which is equal to that measured in cells treated with agonist alone, then AGN 193109's ability to affect the availability of NCPs via promotion of RAR:NCP associations will be silent in this system. This would be an example wherein AGN 193109 has no effect on a coadministered agonist.

Example of Antagonism

The method disclosed in the above generalized example for determining the effect of AGN 193109 coadministered with a retinoid agonist is exemplified by the procedure described under Example 7. CV-1 cells cotransfected with one of the three retinoic acid receptors and the retinoid agonist inducible MTV-TREp-Luc reporter construct were dosed with either ethanol (control, group 1), AGN 193109 at final concentrations of from 10^{-9} to 10^{-6} M (group 2), AGN 193109 at final concentrations of from 10^{-9} to 10^{-6} M coadministered with retinoic acid at 10^{-8} M (group 3), or retinoic

acid (10^{-8} M, group 4). Comparison of the luciferase activity of group 1 with that of group 4 allowed determination of the level of retinoid agonist induced expression of the luciferase reporter gene in the absence of added AGN 193109. Comparison of luciferase reporter gene expression in cells of group 3 with that measured in cells of group 4 indicated that AGN 193109 behaved as an antagonist of the retinoid agonist in this system.

Example of Antagonism

The method disclosed in the generalized example for determining the effect of AGN 193109 coadministered with a retinoid agonist was similarly used to determine in Example 17 that AGN 193109 functioned as an antagonist of a retinoid agonist-mediated repression of EGF-stimulated cellular proliferation in ECE-16-1 transformed cervical epithelial cells. In this procedure, treatments of ECE-16-1 cells included a control sample treated with EGF alone (group 1), a sample treated with the combination of EGF and AGN 193109 at a final concentration of 10^{-6} M (group 2), a sample treated with the combination of EGF and AGN 193109 at final concentrations of from 10^{-10} to 10^{-6} M coadministered with a single dose of the retinoid agonist AGN 191183 at 10^{-8} M (group 3), and a sample treated with the combination of EGF and AGN 191183 at 10^{-8} M (group 4). After three days of treatment, cellular proliferation rates were determined. Determination that the cells had been stimulated to proliferate by EGF was possible because an additional control treatment was included wherein cells were exposed to defined medium that did not contain EGF. Comparison of the number of cells in group I with the number of cells in group 4 allowed for determination that RAR agonist AGN 191183 repressed the EGF-stimulated proliferation of ECE-16-1 cells. Comparison of group 3 with group 4 indicated that AGN 193109 antagonized the activity of the RAR agonist in this system.

Example of Potentiation

The method disclosed in the generalized example for determining the effect of AGN 193109 coadministered with a retinoid agonist was also used in Example 14 to determine that AGN 193109 potentiated the activity of a nuclear receptor agonist in HeLa cells transfected with the 1,25-dihydroxyvitamin D_3 inducible MTV-VDRE-Luc reporter gene. Treatments of transfected cells included vehicle alone (control, group 1), 1,25-dihydroxyvitamin D_3 at final concentrations of from 10^{-10} to 10^{-7} M (group 2), 1,25-dihydroxyvitamin D_3 at final concentrations of from 10^{-8} to 10^{-7} M coadministered with AGN 193109 at a final concentration of either 10^{-8} or 10^{-7} M (group 3), and AGN 193109 as a single agent treatment at a final concentration of either 10^{-8} or 10^{-7} M (group 4). Comparison of the luciferase activity measured in group 1 (control) cells with that of group 2 cells allowed for determination that 1,25-dihydroxyvitamin D_3 stimulated luciferase activity was dose-dependent. Comparison of luciferase activity measured in cells of group 4 (AGN 193109 single agent treatment) with that measured in cells of group 3 (AGN 193109 coadministration) similarly allowed for determination of dose-dependent 1,25-dihydroxyvitamin D_3 stimulated luciferase activity in the presence of a given concentration of AGN 193109. In this instance, the zero value represented the luciferase activity in cells treated with AGN 193109 alone (group 4). Such a dosing regimen allowed for comparison of three 1,25-dihydroxyvitamin D_3 dose response curves. Comparison of the dose response curve of 1,25-dihydroxyvitamin D_3 in the absence of AGN 193109 with the curve representing coadministration of AGN 193109 (either 10^{-8} or 10^{7} M) demonstrated potentiation of the agonist activity as evidenced by a leftward shift in the half-maximal response.

Example of Potentiation

The method disclosed in the generalized example for determining the effect of AGN 193109 coadministered with a retinoid agonist was further used to determine in Example 19 that AGN 193109 potentiated the antiproliferative activity of an RAR agonist in primary cultures of human retinal pigment epithelium cells. Treatments of cells included: ethanol vehicle alone (group 1), retinoic acid at final concentrations of from 10^{-10} to 10^{-6} M (group 2), retinoic acid at final concentrations of from 10^{-10} to 10^{-6} M coadministered with 10^{-6} M AGN 193109 (group 3), and AGN 193109 alone at final concentrations of from 10^{-10} to 10^{-6} M (group 4). Comparison of assay results obtained using cells of groups 1 and 2 allowed for determination of the dose dependent inhibition of proliferation of these cells by retinoic acid. Similarly, comparison of results obtained using cells of group 3 with those of group 1 allowed for determination of the dose dependent inhibition of proliferation of these cells by retinoic acid in the presence of coadministered AGN 193109. Group 4 demonstrated the inability of AGN 193109 to substantially alter the proliferation rate of these cells when used as a single treatment agent. Comparison of the dose response curves of retinoic acid mediated repression of cellular proliferation generated in groups 2 and 3 provided the basis for the conclusion that AGN 193109 sensitized primary RPE cells to the antiproliferative effects of the RAR agonist, thereby potentiating the activity of the RAR agonist.

As indicated above, Agarwal et al., in *Cancer Res.* 54:2108 (1994)), showed that CaSki cell growth, unlike the growth of HPV immortalized ECE-16-1 cells, was not inhibited by treatment with retinoid agonists. As disclosed herein, we unexpectedly found that CaSki cell growth was inhibited by AGN 193109 in the absence of a retinoid agonist. The following Example illustrates how AGN 193109 can be used to inhibit the growth of CaSki cell tumors in vivo.

EXAMPLE 22

Inhibition of CaSki Cell Tumor Growth in Nude Mice Following Administration of AGN 193109

1×10^{6} CaSki cells are injected into each of a panel of nude mice. Tumor formation is assessed using techniques that will be familiar to one having ordinary skill in the art. After injection, mice are randomly divided into control and test groups. The control group receives a placebo. The test group is administered with AGN 193109. Animals administered with the placebo receive intragastric intubation of corn oil. The test animals receive 20 μMol/kg AGN 193109 in corn oil daily for the period of the treatment. Tumor volume is measured in cubic milliliters using graduated calipers. Tumor volume is plotted as function of time. Mice receiving AGN 193109 exhibit tumors which are significantly reduced in their growth rate as compared to tumors in control mice as judged by tumor size and number over the period of the study. This result provides an in vivo demonstration that AGN 193109 inhibits the growth of an advanced cervical carcinoma that is resistant to therapy comprising administration of a retinoid agonist.

As indicated above, CaSki cells are a model of cervical tumors that are not responsive to retinoid agonist therapy. However, herein we have disclosed that CaSki cell growth was inhibited by AGN 193109 in the absence of treatment with a retinoid agonist. The ability of AGN 193109 to inhibit the proliferation of CaSki cells suggested that AGN 193109 could be used to therapeutically treat cervical carcinomas that are insensitive to retinoid agonist therapy. The following Example illustrates one method that can be used to assess the therapeutic potential of AGN 193109 in the treatment of a cervical carcinoma.

EXAMPLE 23

Assessing the Therapeutic Potential of AGN 193109 in Patients Having Cervical Carcinoma

A patient presenting with an advanced cervical carcinoma is first identified. A cervical biopsy is obtained according to methods that will be familiar to one having ordinary skill in the art. Cells from the explanted tumor are propagated in tissue culture according to standard techniques to provide cell numbers sufficient to allow division into three sample groups. Culture conditions described by Agarwal et al. in *Cancer Res.* 54:2108 (1994) are employed for this purpose. The first group is reserved as a control and receives vehicle alone (ethanol). The second group is treated with the RAR agonist retinoic acid at a concentration of from 10^{-10} to 10^{-6} M. The third group is treated with AGN 193109 at doses ranging from 10^{-10} to 10^{-6} M. Cells are fed with fresh growth medium daily and are provided with the retinoids described above as appropriate for each sample group. Cells are counted after three days using an electric cell counter. Comparison of the number of cells in control cultures with the number of cells in retinoic acid treated cultures indicates the RAR agonist does not substantially inhibit the growth rate of the cultured cervical carcinoma cells. In contrast, cells treated with AGN 193109 exhibit a dose-dependent decrease in cell number when compared with cell counts in the control group. This result, wherein AGN 193109 treatment inhibits cultured cervical carcinoma cell proliferation, indicates that AGN 193109 will be a useful therapeutic agent for treating cervical carcinoma patients having metastatic disease.

Cervical carcinoma patients having undergone surgery for the removal of primary tumors and who present with metastatic disease are enlisted in a randomized clinical study seeking to demonstrate the therapeutic benefit of AGN 193109 in this indication. Patients are divided into two groups. The first group is a control group while members of the second group are treated with AGN 193109. AGN 193109 is combined with a pharmaceutically acceptable excipient to produce a composition suitable for systemic administration, all according to techniques that will be familiar to one having ordinary skill in the art. The control group is administered a placebo formulation and the experimental group is administered with the formulation containing the AGN 193109 negative hormone. Dosing of patients is at the maximum tolerated dose and is performed every other day for a period of from three months to one year. The outcome of the study is quantified via measurement of disease-free survival over time. Individuals receiving AGN 193109 display a significant increase in disease-free survival, including a disproportionate number of patients displaying complete remission of their metastatic disease. This result indicates that AGN 193109 has therapeutic utility for in vivo treatment of cervical carcinomas that are unresponsive to the antiproliferative effects of retinoid agonists, such as retinoic acid.

As disclosed above, AGN 193109 potentiated the antiproliferative activity of RAR agonists in primary cultures of human retinal pigment epithelium cells. Accordingly, coadministration of AGN 193109 with an RAR agonist in vivo is reasonably expected to increase the therapeutic index of the agonist because a lesser amount of the RAR agonist will be required to obtain the same therapeutic endpoint. Additionally, AGN 193109 has been demonstrated to sensitize primary cultures of human retinal pigment epithelium cells to the antiproliferative effects of glucocorticoid and thyroid hormone receptor agonists. The following rabbit model of PVR will be utilized in two separate studies to demonstrate the increased therapeutic index obtained via coadministration of AGN 193109 with an RAR agonist (13-cis retinoic acid) or a thyroid hormone receptor agonist, respectively. Notably, the rabbit model of retinal redetachment published by Sen et al. in *Arch. Opthalmol.* 106:1291 (1988), has been used to demonstrate that retinoid agonists which inhibit proliferation of primary RPE cells in vitro also inhibit the frequency of retinal detachment in vivo (Araiz et al. *Invest. Opthalmol.* 34:522 (1993)). Thus, with respect to their use as therapeutics in the prevention of retinal detachment, a correlation between the in vitro and in vivo activities of retinoid agonists has already been established. The following Examples illustrate how AGN 193109 can be used in therapeutic applications directed at 5 preventing retinal detachment.

EXAMPLE 24

Use of AGN 193109 to Increase the Therapeutic Potential of Steroid Superfamily Receptor Agonists in the Treatment of Proliferative Vitreoretinopathy (PVR)

In a first study, human RPE cells are injected into the vitreous cavity of rabbit eyes according to the method described by Sen et al. in *Arch Opthalmol.* 106:1291 (1988). After intravitreal injection, the rabbits are divided into five groups. The first group (control) will receive vehicle alone by intravitreal injection. The second group receives retinoic acid as single agent treatment (100 μg) by intravitreal injection. The third group receives AGN 193109 as a single agent treatment (100 μg) by intravitreal injection. The fourth group receives by intravitreal injection the RAR agonist (retinoic acid) at a dose one-tenth the amount administered to group 2 (10 μg). The fifth group receives the combination of AGN 193109 (100 μg) and retinoic acid (10 μg) by intravitreal injection. Animals receive a single intravitreal injection of the appropriate treatment one day after intravitreal injection of human RPE cells. Rabbits are examined by indirect ophthalmoscopy on days 7, 14 and 28, and are graded for the frequency and severity of tractional retinal detachment. Rabbits from the group injected with 100 μg retinoic acid exhibit a significantly reduced frequency and severity of retinal detachment compared to control rabbits or rabbits receiving either AGN 193109 or retinoic acid (10 μg) alone. Rabbits in the group administered with the combination of AGN 193109 and retinoic acid (10 μg) exhibit significantly reduced frequency and severity of retinal detachment as compared to those in groups either control, AGN 193109 or retinoic acid (10 μg). This result demonstrates that AGN 193109 improves the therapeutic index of the RAR agonist retinoic acid in an in vivo model of PVR.

In a second study, rabbits are first provided with an injection of human RPE cells into the vitreous cavity of the eye, and then divided into four groups. The first group

(control) receives vehicle alone by intravitreal injection. The second group receives thyroid hormone as single agent treatment (100 μg) by intravitreal injection. The third group is administered with AGN 193109 as a single agent treatment (100 μg) by intravitreal injection. The fourth group is administered with the combination of AGN 193109 (100 μg) and thyroid hormone (100 μg). Rabbits are examined by indirect ophthalmoscopy on days 7, 14 and 28, and graded for the frequency and severity of tractional retinal detachment. Comparison of the frequency and severity of retinal detachment in the four groups demonstrates that single agent treatment with either AGN 193109 or thyroid hormone does not inhibit retinal detachment when compared with control rabbits. In contrast, the group of rabbits administered with the combination of AGN 193109 and thyroid hormone exhibit significantly reduced incidence and severity of retinal detachment. This result demonstrates that AGN 193109 improves the therapeutic index of thyroid hormone in an in vivo model of PVR.

The following Example illustrates how AGN 193109 can be used to enhance the therapeutic index of an RAR agonist used to treat human patients following retinal reattachment surgery.

EXAMPLE 25

Increasing the Therapeutic Index of RAR Agonist 13-cis Retinoic Acid

A population of adult volunteers having retinal detachment resulting from PVR is first identified. Individuals undergo surgical repair of the detachments using techniques that are standard in the art. The patients are then divided into five groups. The control group consists of patients who undergo surgical repair of the retinal detachment and do not receive any retinoid compound. The second group receives 40 mg oral 13-cis retinoic acid twice daily for four weeks postoperatively. The third group receives 40 mg oral AGN 103109 twice daily for four weeks postoperatively. The fourth group receives 4 mg oral 13-cis retinoic acid twice daily for four weeks postoperatively. The fifth group receives 40 mg oral AGN 193109 in combination with 4 mg oral 13-cis retinoic acid twice daily for four weeks postoperatively. The treatment protocol and assessment of drug efficacy is performed essentially as described by Fekrat et al. in *Ophthalmology* 102:412 (1995).

The frequency and severity of retinal redetachment in postoperative patients in all five groups is monitored over a period of nine months using ophthalmologic examination techniques that will be familiar to those of ordinary skill in the art. Patients receiving 40 mg oral 13-cis retinoic acid exhibit significantly reduced incidence of retinal redetachment when compared with control patients, patients receiving 4 mg oral 13-cis retinoic acid twice daily or patients receiving 40 mg oral AGN 193109 twice daily. Examination of the patient group receiving the combination of 40 mg oral AGN 193109 and 4 mg oral 13-cis retinoic acid twice daily for four weeks postoperatively demonstrates the therapeutic outcome in this patient group is equal to or better than those patients receiving 40 mg oral 13-cis retinoic acid twice daily for four weeks postoperatively. This result demonstrates that the AGN 193109 negative hormone improves the therapeutic index of an RAR agonist by virtue of decreasing the frequency and severity of retinal redetachment in PVR patients.

Generalized Assay for Identifying Nuclear Receptor Negative Hormones

We have demonstrated above that AGN 193109 can function as a negative hormone capable of repressing the basal transcriptional activity of RAR nuclear receptors. Further, we have described an assay using CV-1 cells co-transfected with the ERE-tk-Luc luciferase reporter plasmid and the ER-RXR-α and RAR-γ-VP-16 receptor expression plasmids for distinguishing RAR ligands that are simple antagonists from those having negative hormone activity.

We have concluded that RAR negative hormones mediate repression of RAR-mediated transcriptional activity by promoting increased interaction between the RAR and NCPs. Further, we have demonstrated that AGN 193109 can potentiate the effects of agonists of other nuclear receptors in a manner consistent with the mutual sharing of NCPs between members of the steroid superfamily of nuclear receptors. As such, ligands can be designed and screened to identify compounds having negative hormone activity at these non-RAR nuclear receptors.

Our method of RAR negative hormone screening based on the use of CV-1 cells co-transfected with the ERE-tk-Luc luciferase reporter plasmid and the ER-RXR-α and RAR-γ-VP-16 receptor expression plasmids can be adapted generally such that the RAR-γ moiety of the RAR-γ-VP-16 plasmid is converted to that of peroxisome proliferator-activated receptors (PPAR), vitamin D receptor (VDR), thyroid hormone receptor (T3R) or any other steroid superfamily nuclear receptor capable of heterodimerizing with RXR. CV-1 cells co-transfected with such plasmids would express high basal levels of luciferase activity. Ligands capable of binding the ligand binding domain of the receptor substituted for the RAR-γ moiety can be easily screened for negative hormone activity by measuring their ability to repress luciferase activity.

For steroid superfamily nuclear receptors that do not heterodimerize with RXR (e.g., glucocorticoid and estrogen receptors) the same end result can be achieved using GR-VP-16 or ER-VP-16 receptors and a luciferase reporter plasmid consisting of the appropriate glucocorticoid or estrogen response element fused to a heterologous promoter element and luciferase or other reporter gene. An essential feature of a generalized negative hormone screening assay is the inclusion of at least the ligand binding domain of the particular nuclear receptor for which inverse agonists are to be screened and a method for localizing the nuclear receptor ligand binding domain to the promoter of a reporter gene. This could be achieved using the receptors's natural DNA binding site, or alternatively by construction of a chimeric receptor having a heterologous DNA binding domain and corresponding use of a reporter gene which is under control of a DNA regulatory element which is recognized by the heterologous DNA binding domain. In a preferred embodiment, the plasmid expressing the nuclear receptor for which inverse agonists are to be screened would express this nuclear receptor as a fusion protein containing a constitutive activation domain, such as the HSV VP-16 activation domain, in order to provide allow high basal activity. This high basal activity would effectively increase assay sensitivity, thereby allowing analysis of nuclear receptor ligands which repress basal transcriptional activity in the absence of added nuclear receptor agonist.

The following Example illustrates one method that can be used to screen for compounds having negative hormone activity at the thyroid hormone receptor.

EXAMPLE 26

Method of Identifying Thyroid Hormone Receptor Negative Hormones

CV-1 cells are co-transfected with the luciferase reporter plasmid ERE-tk-Luc and the plasmids ER-RXR-α and T3R-

VP-16. T3R-VP-16 is identical to the plasmid RAR-γ-VP-16, except the RAR-γ moiety of RAR-γ-VP-16 has been substituted by the thyroid hormone receptor cDNA. As such, T3R-VP-16 expresses a fusion protein containing the activation domain of HSV VP-16 in frame with the N-terminus of the thyroid hormone receptor. Standard transfection and cell culture methods are employed for this purpose. After transfection, cells are rinsed and fed with growth medium containing 10% fetal calf serum which has been extracted with activated charcoal. Cells are treated with vehicle alone (ethanol), thyroid hormone (10^{-9} to 10^{-10} M), or compound TR-1 (10^{-9} to 10^{-6} M). TR-1 is a synthetic thyroid hormone receptor ligand which exhibits strong affinity for the thyroid hormone receptor in competition binding studies, but which does not activate transfected thyroid hormone receptor in transient cotransfection transactivation assays using a thyroid hormone responsive reporter gene and a thyroid hormone receptor expression plasmid. Further, TR-1 is capable of antagonizing thyroid hormone mediated transactivation and as such is a thyroid receptor antagonist.

Analysis of luciferase activity from CV-1 cell transfected with ERE-tk-Luc, ER-RXRα and T3R-VP-16 demonstrates a high basal level of luciferase reporter activity in vehicle-treated cells. Cells treated with thyroid hormone show a slight increase of luciferase activity in a dose dependent manner. Cells treated with TR-1 exhibit a dose dependent decrease in luciferase activity. This indicates that TR-1 exhibits thyroid receptor inverse agonist activity, presumably due to the increased interaction of a NCP with the thyroid hormone receptor.

The proliferation rate of human primary retinal pigment epithelium cells is repressed by treatment with RAR agonists. The therapeutic value of this observation has been demonstrated in post-operative use retinoid therapy after retinal reattachment surgery. We have above demonstrated the AGN 193109 RAR negative hormone can sensitize primary RPE cells to the antiproliferative effect of ATRA and 13-cis retinoic acid in coadministration procedures. Further, AGN 193109 was also shown to sensitize RPE cells to the antiproliferative effects of other nuclear receptor agonists. More specifically, AGN 193109 sensitized RPE cells to the antiproliferative effects of the glucocorticoid agonist, dexamethasone, and the thyroid hormone agonist 3,3',5-triiodothyronine, T3. This data was consistent with our working model wherein AGN 193109 modulated the availability of NCPs that were shared between the members of the nuclear receptor family. Treatment of RPE cells with the thyroid hormone receptor inverse agonist TR-1 will similarly alter the availability of shared NCPs such that coadministration with a non-thyroid receptor agonist, such as the RAR agonist 13-cis retinoic acid will lead to an increased antiproliferative effect upon the RPE cultures as compared to 13-cis retinoic acid as a single agent treatment.

The following Example illustrates one method that can be used to render primary RPE cells more sensitive to the antiproliferative activity of an RAR agonist. Notably, this Example further illustrates how the activity of RAR agonists can be potentiated by coadministration with a negative hormone.

EXAMPLE 27

Sensitizing Primary Retinal Pigment Epithelium Cells to the Antiproliferative Effects of RAR Agonists by Coadministration of the TR-1 Thyroid Hormone Inverse Agonist

Human primary RPE cells are obtained and cultured according to standard methods. The cultured cells are divided into four groups and treated as follows. Group 1 receives vehicle alone (ethanol). Group 2 is treated with 13-cis retinoic acid at concentrations ranging from 10^{-11} to 10^{-6} M. Group 3 is treated with the thyroid hormone inverse agonist TR-1 at concentrations ranging from 10^{-11} to 10^{-1} M. Group 4 is co-treated with 13-cis retinoic acid at concentrations ranging from 10^{-11} to 10^{-6} M TR-1. Cells are refed with fresh growth medium and re-treated with the appropriate compound every two days for a total of five days of treatment. The proliferation rate over the duration of the experiment is quantitated via measurement of the cell number in the cultures using an electric cell counter.

TR-1 treated cells (Group 3) exhibits rates of cellular proliferation which are essentially the same as control (Group 1) cells and there is no effect of this inverse agonist upon the measured growth rate of the cultures. Cells treated with 13-cis retinoic acid (Group 2) exhibit a dose dependent decrease in cell number. Comparison of the dose dependent decrease in cellular proliferation of Group 4 cells (13-cis RA and TR-1 coadministration) with that obtained in Group 3 demonstrates the ability of TR-1 thyroid hormone receptor inverse agonist coadministration to sensitize RPE cultures to the antiproliferative effect of 13-cis retinoic acid as measured by the shift in the dose response curve of this RAR agonist to the left in Group 4 as compared to Group 2 cells.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 9

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCACCCATGG CAAATTCCAT GGCA 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

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(A) LENGTH: 20 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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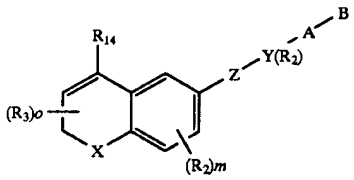


wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C—≡C—
—N=N—,
—N=CR_1—,
—CR_1=N—,
—(CR_1=CR_1)$_n$,—where n' is an integer having the value 0–5,
—CO—NR_1—,
—CS—NR_1—,
—NR_1—CO,
—NR_1—CS,
—COO—,
—OCO—;
—CSO—;
—OCS—;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, —CH_2OH, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$—, —COR_7, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is (R_{15})$_r$-phenyl, (R_{15})$_r$-naphthyl, or (R_{15})$_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroa-

toms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

2. A compound of claim 1 where Y is pyridyl, thienyl or furyl.

3. A compound of claim 1 where Y is pyridyl.

4. A compound of claim 1 where R_2 is H, F, or CF_3.

5. A compound of claim 1 where R_3 is H or methyl.

6. A compound of claim 1 where R_{14} is $(R_{15})_r$- phenyl.

7. A compound of claim 1 where R_{14} is $(R_{15})_r$- heteroaryl.

8. A compound of claim 7 where R_{14} is $(R_{15})_r$- heteroaryl where the heteroaryl group is a 5 or six membered ring having 1 or 2 heteroatoms.

9. A compound of claim 8 where the heteroaryl group is selected from 2-pyridyl, 3-pyridyl, 2-thienyl and 2-thiazolyl.

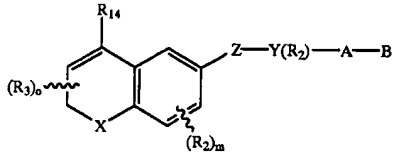
10. A compound of claim 1 where the R_{15} group is H, CF_3, F, lower alkyl, lower alkoxy, hydroxy or chlorine.

11. A compound of claim 1 where Z is —C≡C—.

12. A compound of claim 1 where Z is $—CS_1—NR—$.

13. A compound of claim 1 where X is S.

14. A compound of the formula



wherein X is S, O NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C≡C—

—N=N—,

$—N=CR_1—$,

$—CR_1=N—$,

$—(CR_1=CR_1)_{n'}—$where n' is an integer having the value 0–5,

$—CO—NR_1—$,

$—CS—NR_1—$,

$—NR_1—CO$,

$—NR_1—CS$,

—COO—,

—OCO—;

—CSO—;

—OCS—;

Y is heteroaryl selected from a group consisting of thienyl and furyl, said thienyl and furyl groups being optionally substituted with one or two R_2 group;

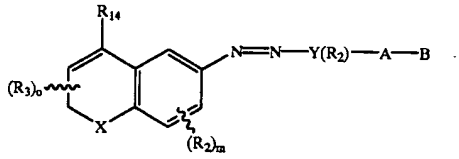
A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, COR_7, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

15. A compound of the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

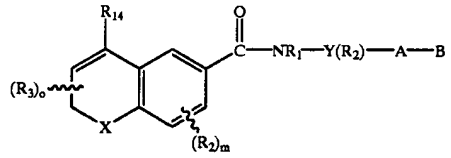
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where

the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11}, is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- -naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

16. A compound of the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

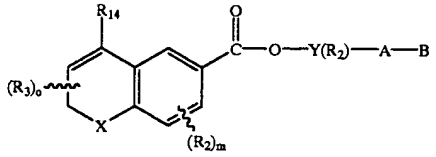
A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

17. A compound of the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

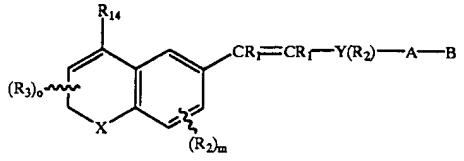
A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, NH(R8), COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

18. A compound of the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons; R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

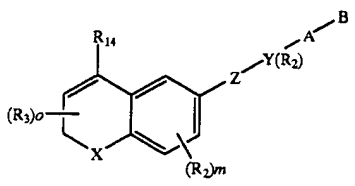
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

19. The method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, $RAR_\beta$and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula:

Formula 1



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C≡C—

—N=N—,

—N=CR_1—,

—CR_1=N—,

—$(CR_1=CR_1)_n$—where n' is an integer having the value 0–5,

—CO—NR_1—,

—CS—NR_1—,

—NR_1—CO,

—NR_1—CS,

—COO—,

—OCO—;

—CSO—;

—OCS—;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an

alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

20. A method of claim **19** where in the formula of the antagonist or negative hormone Y is pyridyl, thienyl or furyl.

21. A method of claim **19** where in the formula of the antagonist or negative hormone Y is pyridyl.

22. A method of claim **19** where in the formula of the antagonist or negative hormone R_2 is H, F, or CF_3.

23. A method of claim **19** where in the formula of the antagonist or negative hormone R_3 is H or methyl.

24. A method of claim **19** where in the formula of the antagonist or negative hormone R_{14} is $(R_{15})_r$- phenyl.

25. A method of claim **19** where in the formula of the antagonist or negative hormone R_{14} is $(R_{15})_r$- heteroaryl.

26. A method of claim **25** where in the formula of the antagonist or negative hormone R_{14} is $(R_{15})_r$- heteroaryl where the heteroaryl group is a 5 or six membered ring having 1 or 2 heteroatoms.

27. A method of claim **26** where in the formula of the antagonist the heteroaryl group is selected from 2-pyridyl, 3-pyridyl, 2-thienyl and 2-thiazolyl.

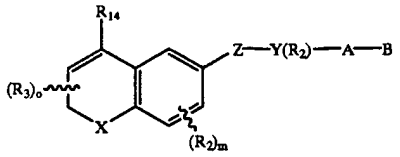
28. A method of claim **19** where in the formula of the antagonist or negative hormone the R_{15} group is H, CF_3, F, lower alkyl, lower alkoxy, hydroxy or chlorine.

29. A method of claim **19** where in the formula of the antagonist or negative hormone Z is —C≡C—.

30. A method of claim **19** where in the formula of the antagonist or negative hormone Z is —COO—.

31. A method of claim **19** where in the formula of the antagonist or negative hormone X is S.

32. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, $RAR_\beta$and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Z is —C═C—

—N═N—,

—N═CR_1—,

—CR_1═N—,

—$(CR_1═CR_1)_{n'}$—where n' is an integer having the value 0–5,

—CO—NR_1—,

—CS—NR_1—,

—NR_1—CO,

—NR_1—CS,

—COO—,

—OCO—;

—CSO—;

—OCS—;

Y is heteroaryl selected from a group consisting of thienyl and furyl, said thienyl and furyl groups being optionally substituted with one or two R_2 group;

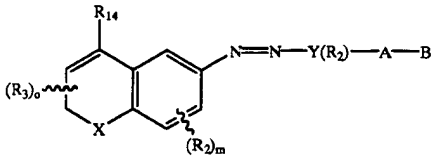
A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

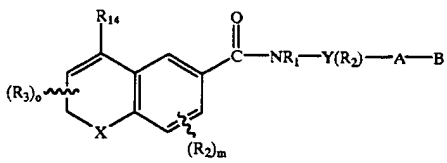
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, COOR8, $CONR_9R_{10}$, —CH_2OH, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, —COR_7, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$—phenyl, $(R_{15})_r$-naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

33. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, RAR_β and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula





wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

34. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, RAR_β and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula

wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

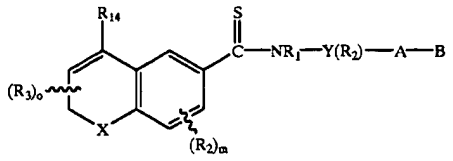
A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, COOR8, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

35. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, RAR_β and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

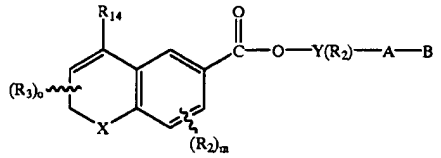
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

36. A method in accordance with claim **35** wherein in the formula of the antagonist or negative hormone R_1 is H.

37. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, RAR_β and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

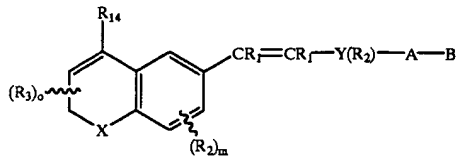
B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_{12} is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_{14} is $(R_{15})_r$- phenyl, $(R_{15})_r$- naphthyl, or $(R_{15})_r$- heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

38. A method of treating a pathological condition in a mammal, said condition associated with a retinoic acid receptor activity, said method comprising administering to said mammal a retinoid antagonist or negative hormone capable of binding to a retinoic acid receptor subtype selected from the group consisting of RAR_α, RAR_β and RAR_γ, said antagonist or negative hormone being administered in an amount pharmaceutically effective to provide a

therapeutic benefit against said pathological condition in said mammal and wherein the negative hormone or antagonist has the formula



wherein X is S, O, NR' where R' is H or alkyl of 1 to 6 carbons;

R_2 is hydrogen, lower alkyl of 1 to 6 carbons, F, Cl, Br, I, CF_3, fluoro substituted alkyl of 1 to 6 carbons, OH, SH, alkoxy of 1 to 6 carbons, or alkylthio of 1 to 6 carbons;

R_3 is hydrogen, lower alkyl of 1 to 6 carbons or F;

m is an integer having the value of 0–3;

o is an integer having the value of 0–3;

Y is heteroaryl selected from a group consisting of pyridyl, thienyl, furyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiazolyl, oxazolyl, imidazolyl and pyrrazolyl, said heteroaryl groups being optionally substituted with one or two R_2 group;

A is $(CH_2)_q$ where q is 0–5, lower branched chain alkyl having 3–6 carbons, cycloalkyl having 3–6 carbons, alkenyl having 2–6 carbons and 1 or 2 double bonds, alkynyl having 2–6 carbons and 1 or 2 triple bonds;

B is hydrogen, COOH or a pharmaceutically acceptable salt thereof, $COOR_8$, $CONR_9R_{10}$, $—CH_2OH$, CH_2OR_{11}, CH_2OCOR_{11}, CHO, $CH(OR_{12})_2$, $CHOR_{13}O$, $—COR_7$, $CR_7(OR_{12})_2$, $CR_7OR_{13}O$, or tri-lower alkylsilyl, where R_7 is an alkyl, cycloalkyl or alkenyl group containing 1 to 5 carbons, R_8 is an alkyl group of 1 to 10 carbons or trimethylsilylalkyl where the alkyl group has 1 to 10 carbons, or a cycloalkyl group of 5 to 10 carbons, or R_8 is phenyl or lower alkylphenyl, R_9 and R_{10} independently are hydrogen, an alkyl group of 1 to 10 carbons, or a cycloalkyl group of 5–10 carbons, or phenyl or lower alkylphenyl, R_{11} is lower alkyl, phenyl or lower alkylphenyl, R_2 is lower alkyl, and R_{13} is divalent alkyl radical of 2–5 carbons, and

R_4 is $(R_{15})_r$—phenyl, $(R_{15})_r$—naphthyl, or $(R_{15})_r$-heteroaryl where the heteroaryl group has 1 to 3 heteroatoms selected from the group consisting of O, S and N, r is an integer having the values of 0–5, and

R_{15} is independently H, F, Cl, Br, I, NO_2, $N(R_8)_2$, $NH(R_8)$, COR_8, $NR_8CON(R_8)_2$, OH, $OCOR_8$, OR_8, CN, an alkyl group having 1 to 10 carbons, fluoro substituted alkyl group having 1 to 10 carbons, an alkenyl group having 1 to 10 carbons and 1 to 3 double bonds, alkynyl group having 1 to 10 carbons and 1 to 3 triple bonds, or a trialkylsilyl or trialkylsilyloxy group where the alkyl groups independently have 1 to 6 carbons.

* * * * *

US006001847A

United States Patent [19]

Daugan et al.

[11] Patent Number: 6,001,847

[45] Date of Patent: Dec. 14, 1999

[54] CHEMICAL COMPOUNDS

[75] Inventors: Alain Claude-Marie Daugan, Les Ulis, France; Richard Frederic LaBaudiniere, Collegeville, Pa.

[73] Assignee: ICOS Corporation, Bothell, Wash.

[21] Appl. No.: 08/981,966

[22] PCT Filed: Jul. 11, 1996

[86] PCT No.: PCT/EP96/03023

§ 371 Date: Jun. 8, 1998

§ 102(e) Date: Jun. 8, 1998

[87] PCT Pub. No.: WO96/32003

PCT Pub. Date: Oct. 17, 1996

[30] Foreign Application Priority Data

Jul. 14, 1995 [GB] United Kingdom 9414473

[51] Int. Cl.[6] A01N 43/42; A01N 43/58; C07D 239/00; C07D 471/00

[52] U.S. Cl. 514/287; 544/247; 544/343; 546/64; 546/85; 546/86; 546/87; 514/92; 514/249; 514/250

[58] Field of Search 544/247, 343; 546/64, 85, 86, 87; 514/92, 287, 249, 250

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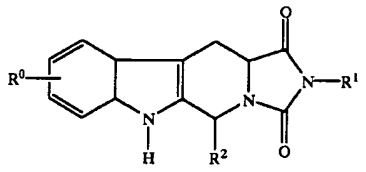
[57] ABSTRACT

A compound of formula



and salts and solvates thereof, wherein R^0, R^1, and R^2 are defined in the specification. A compound of the present invention is a potent and selective inhibitor of cGMP-specific PDE and has utility in a variety of therapeutic areas where such inhibition is beneficial.

15 Claims, No Drawings

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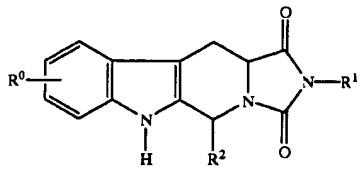
CHEMICAL COMPOUNDS

This application is a priority 371 of PCT/EP96/03023 filed Jul. 11, 1997.

This invention relates to a series of tetracyclic derivatives, to processes for their preparation, pharmaceutical compositions containing them, and their use as therapeutic agents. In particular, the invention relates to tetracyclic derivatives which are potent and selective inhibitors of cyclic guanosine 3', 5'-monophosphate specific phosphodiesterase (cGMP specific PDE) having utility in a variety of therapeutic areas where such inhibition is thought to be beneficial, including the treatment of cardiovascular disorders.

Thus, according to a first aspect, the present invention provides compounds of formula (I)

(I)



and salts and solvates (e.g. hydrates) thereof, in which:

R^0 represents hydrogen, halogen or C_{1-6} alkyl;

R^1 is selected from the group consisting of:

(a) hydrogen;

(b) C_{1-6}alkyl optionally substituted by one or more substituents selected from phenyl, halogen, $—CO_2R^a$ and $—NR^aR^b$;

(c) C^{3-6}cycloalkyl;

(d) phenyl; and

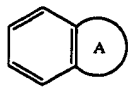
(e) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur, and being optionally substituted by one or more C_{1-6}alkyl, and optionally linked to the nitrogen atom to which R^1 is attached via C_{1-6}alkyl;

R is selected from the group consisting of:

(f) C_{3-6}cycloalkyl;

(g) phenyl optionally substituted by one or more substituents selected from $—OR^a$, $—NR^aR^b$, halogen, hydroxy, trifluoromethyl, cyano and nitro;

(h) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur; and



(i) a bicyclic ring attached to the rest of the molecule via one of the benzene ring carbon atoms and A is a 5- or 6-membered heterocyclic ring as defined in point (h); and

R^a and R^b independently represent hydrogen or C_{1-6}alkyl.

The term "C_{1-6}alkyl" as used herein denotes any straight or branched alkyl chain containing 1 to 6 carbon atoms, and includes methyl, ethyl, n-propyl, iso-propyl, n-butyl, pentyl, hexyl and the like.

The term "halogen" as used herein denotes fluorine, chlorine, bromine and iodine.

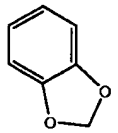
A particular group of compounds according to formula (I) are those wherein R^0 represents any of hydrogen, methyl, bromine and fluorine, although of course the definition of R^0 given in formula (I) includes within its scope other C_{1-6}alkyl and halogen groups.

Aptly, R^1 may represent a substituent selected from methyl, ethyl optionally substituted by one or more chlorine atoms, butyl, cyclohexyl and benzyl.

Other suitable R^1 substituents include hydrogen; cycloalkyl groups, such as cyclopropyl; C_{1-6}alkyl, typically ethyl or propyl, substituted by an $—NR^aR^b$ substituent, such as a dimethylamino substituent; phenyl optionally linked to the nitrogen atom to which R^1 is attached via a C_{1-6}alkyl chain, such as ethyl or the like; and C_{1-6}alkyl, e.g. methyl, substituted by $—CO_2R^a$, such as $—CH_2CO_2Et$ or the like.

Suitable heterocyclic rings within the definition of R^1 include pyridyl, morpholinyl, piperazinyl, pyrrolidinyl and piperidinyl. Generally such heterocyclic rings are linked to the nitrogen atom to which R^1 is attached via a C_{1-6}alkyl chain, more appropriately a C_{1-4}alkyl chain.

A particularly apt substituent represented by R^2 is



Other suitable R^2 substituents include thienyl, pyridyl, furyl and phenyl, wherein phenyl can be substituted by one or more substituents selected from $—OR^a$ (e.g. methoxy), $—NR^aR^b$ (e.g. dimethylamino), halogen (in particular chlorine or fluorine), hydroxy, trifluoromethyl, cyano and nitro.

Alternatively, R^2 may represent a suitable C_{3-6}cycloalkyl group, such as cyclohexyl or the like.

The pharmaceutically acceptable salts of the compounds of formula (I) which contain a basic centre are acid addition salts formed with pharmaceutically acceptable acids. Examples include the hydrochloride, hydrobromide, sulphate or bisulphate, phosphate or hydrogen phosphate, acetate, benzoate, succinate, fumarate, maleate, lactate, citrate, tartrate, gluconate, methanesulphonate, benzenesulphonate and p-toluenesulphonate salts. Compounds of the formula (I) can also provide pharmaceutically acceptable metal salts, in particular alkali metal salts, with bases. Examples include the sodium and potassium salts.

It is to be understood that the present invention covers all appropriate combinations of particular and preferred groupings hereinabove.

Particular individual compounds of the invention include:

Cis-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6, 11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6, 11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione;

Cis-5-(4-methoxyphenyl)-2-methyl-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-ethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-ethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-ethyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-ethyl-5-(2-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-dimethylaminophenyl)-2-ethyl-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-9-methyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-9-bromo-2-butyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(4-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole1,3(2H)-dione;

Trans-2-butyl-5-(4-fluorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-hydroxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(4-trifluoromethylphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(4-cyanophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-cyanophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(4-nitrophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-nitrophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-pyridyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-cyclohexyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-cyclohexyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-cyclohexyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione; Trans-2-cyclohexyl-9-fluoro-5-(4-methoxyphenyl)-5,6, 1 1,1 1 a-tetrahydro-1H-imidazo [1', 5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Cis-2-benzyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

(5R,11aR)-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-(4-hydroxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-(2-chloroethyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-cyclohexyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Cis-2-cyclohexyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-ethoxycarbonylmethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-2-[2-(2-pyridyl)-ethyl]-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-cyclopropyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans -2-phenethyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-5-phenyl-2-(2-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-5-phenyl-2-(4-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-2-(3-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-(2-dimethylamino-ethyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-(3-dimethylamino-propyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro -1H-imidazo [1', 5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-(2-morpholin-4-yl-ethyl)-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6] pyrido [3,4-b] indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-2-[3-(4-methyl-piperazin-1-yl)-propyl]-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1, 6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-2-(2-pyrrolidin-1-yl-ethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-2-[2-(1-methyl-pyrrolidin-2-yl)-ethyl]-5,6,11,11a-tetrahydro -1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3 (2H)-dione;

Cis-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3 (2H)-dione;

and pharmaceutically acceptable salts and solvates thereof.

Particularly preferred compounds of the invention are:

(5R,11aR)-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione;

Cis-2-cyclohexyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

and pharmaceutically acceptable salts and solvates thereof.

It has been shown that compounds of the present invention are potent and selective inhibitors of cGMP specific PDE. Thus, compounds of formula (I) are of interest for use in therapy, specifically for the treatment of a variety of conditions where inhibition of cGMP specific PDE is thought to be beneficial.

As a consequence of the selective PDE V inhibition exhibited by compounds of the present invention, cGMP levels are elevated, which in turn can give rise to beneficial anti-platelet, anti-neutrophil, anti-vasospastic, vasodilatory, natriuretic and diuretic activities as well as potentiation of the effects of endothelium-derived relaxing factor (EDRF), nitrovasodilators, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and endothelium-dependent relaxing agents such as bradykinin, acetylcholine and 5-HT_1. The compounds of formula (I) therefore have utility in the treatment of a number of disorders, including stable, unstable and variant (Prinzmetal) angina, hypertension, pulmonary hypertension, congestive heart failure, renal failure, atherosclerosis, conditions of reduced blood vessel patency (e.g. post-percutaneous transluminal coronary angioplasty), peripheral vascular disease, vascular disorders such as Raynaud's disease, inflammatory diseases, stroke, bronchitis, chronic asthma, allergic asthma, allergic rhinitis, glaucoma, erectile dysfunction and diseases characterised by disorders of gut motility (e.g. irritable bowel syndrome).

It will be appreciated that references herein to treatment extend to prophylaxis as well as treatment of established conditions.

It will also be appreciated that 'a compound of formula (I),' or a physiologically acceptable salt or solvate thereof can be administered as the raw compound, or as a pharmaceutical composition containing either entity.

There is thus provided as a further aspect of the invention a compound of formula (I) for use in the treatment of stable, unstable and variant (Prinzmetal) angina, hypertension, pulmonary hypertension, chronic obstructive pulmonary disease, congestive heart failure, renal failure, atherosclerosis, conditions of reduced blood vessel patency, (e.g. post-PTCA), peripheral vascular disease, vascular disorders such as Raynaud's disease, inflammatory diseases, stroke, bronchitis, chronic asthma, allergic asthma, allergic rhinitis, glaucoma, erectile dysfunction or diseases characterised by disorders of gut motility (e.g. IBS).

According to another aspect of the invention, there is provided the use of a compound of formula (I) for the manufacture of a medicament for the treatment of stable, unstable and variant (Prinzmetal) angina, hypertension, pulmonary hypertension, chronic obstructive pulmonary disease, congestive heart failure, renal failure, atherosclerosis, conditions of reduced blood vessel patency, (e.g. post-PTCA), peripheral vascular disease, vascular disorders such as Raynaud's disease, inflammatory diseases, stroke, bronchitis, chronic asthma, allergic asthma, allergic rhinitis, glaucoma, erectile dysfunction or diseases characterised by disorders of gut motility (e.g. IBS).

In a further aspect, the invention provides a method of treating stable, unstable and variant (Prinzmetal) angina, hypertension, pulmonary hypertension, chronic obstructive pulmonary disease, congestive heart failure, renal failure, atherosclerosis, conditions of reduced blood vessel patency, (e.g. post-PTCA), peripheral vascular disease, vascular disorders such as Raynaud's disease, inflammatory diseases, stroke, bronchitis, chronic asthma, allergic asthma, allergic rhinitis, glaucoma, erectile dysfunction or diseases characterised by disorders of gut motility (e.g. IBS) in a human or non-human animal body which comprises administering to said body a therapeutically effective amount of a compound with formula (I).

Compounds of the invention may be administered by any suitable route, for example by oral, buccal, sub-lingual, rectal, vaginal, nasal, topical or parenteral (including intravenous, intramuscular, subcutaneous and intracoronary) administration. Oral administration is generally preferred.

For administration to man in the curative or prophylactic treatment of the disorders identified above, oral dosages of a compound of formula (I) will generally be in the range of from 0.5–800 mg daily for an average adult patient (70 kg). Thus for a typical adult patient, individual tablets or capsules contain from 0.2400 mg of active compound, in a suitable pharmaceutically acceptable vehicle or carrier, for administration in single or multiple doses, once or several times per day. Dosages for intravenous, buccal or sublingual administration will typically be within the range of from 0.1–400 mg per single dose as required. In practice the physician will determine the actual dosing regimen which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case but there can be individual instances in which higher or lower dosage ranges may be merited, and such are within the scope of this invention.

For human use, a compound of the formula (I) can be administered alone, but will generally be administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard phar-

maceutical practice. For example, the compound may be administered orally, buccally or sublingually, in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavouring or colouring agents. Such liquid preparations may be prepared with pharmaceutically acceptable additives such as suspending agents (e.g. methylcellulose, a semi-synthetic glyceride such as witepsol or mixtures of glycerides such as a mixture of apricot kernel oil and PEG-6 esters or mixtures of PEG-8 and caprylic/capric glycerides). A compound may also be injected parenterally, for example intravenously, intramuscularly, subcutaneously or intracoronarily. For parenteral administration, the compound is best used in the form of a sterile aqueous solution which may contain other substances, for example salts, or monosaccharides such as mannitol or glucose, to make the solution isotonic with blood.

Thus, the invention provides in a further aspect a pharmaceutical composition comprising a compound of the formula (I) together with a pharmaceutically acceptable diluent or carrier therefor.

There is further provided by the present invention a process of preparing a pharmaceutical composition comprising a compound of formula (I), which process comprises mixing a compound of formula (I) together with a pharmaceutically acceptable diluent or carrier therefor.

A compound of formula (I) may also be used in combination with other therapeutic agents which may be useful in the treatment of the above-mentioned disease states. The invention thus provides, in another aspect, a combination of a compound of formula (I) together with another therapeutically active agent.

The combination referred to above may conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical compositions comprising a combination as defined above together with a pharmaceutically acceptable diluent or carrier comprise a further aspect of the invention.

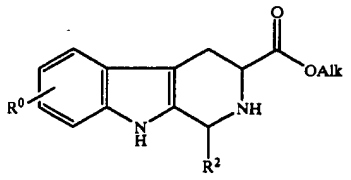
The individual components of such a combination may also be administered either sequentially or simultaneously in separate pharmaceutical formulations.

Appropriate doses of known therapeutic agents for use in combination with a compound of formula (I) will be readily appreciated by those skilled in the art.

Compounds of formula (I) may be prepared by any suitable method known in the art or by the following processes which form part of the present invention. In the methods below R^0, R^1 and R^2 are as defined in formula (I) above unless otherwise indicated.

Thus, a process (A) for preparing a compound of formula (I) comprises reacting a compound of formula (II)

(II)



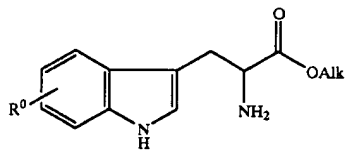
with an isocyanate of formula R^1—N=C=O, in the presence of a suitable organic solvent, such as a ketone solvent, e.g. butanone, acetone or the like, and under reflux for several hours, e.g. 14 to 16 hours. Alk as used herein represents a C_{1-6}alkyl group, e.g. methyl.

Compounds of formula (I) may be prepared as individual enantiomers in two steps from the appropriate enantiomer of formula (III) or as mixtures (e.g. racemates) of either pairs of cis or trans isomers from the corresponding mixtures of either pairs of cis or trans isomers of formula (III).

Individual enantiomers of the compounds of the invention may be prepared from racemates by resolution using methods known in the art for the separation of racemic mixtures into their constituent enantiomers, for example using HPLC (high performance liquid chromatography) on a chiral column such as Hypersil naphthylurea.

A compound of formula (II) may conveniently be prepared from a tryptophan derivative, such as an alkyl ester thereof of formula (III)

(III)



(where Alk is as previously defined) or a salt thereof (e.g. the hydrochloride salt) according to either of the following procedures (a) and (b). Procedure (b) is only suitable for preparing cis isomers of formula (III) and may be particularly suitable for preparing individual cis enantiomers of formula (III) from D- or L-tryptophan alkyl esters as appropriate.

Procedure (a)

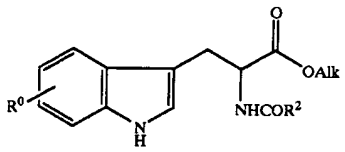
This comprises a Pictet-Spengler cyclisation between a compound of formula (III) and an aldehyde R^2CHO. The reaction may conveniently be effected in a suitable solvent such as a halogenated hydrocarbon (e.g. dichloromethane) or an aromatic hydrocarbon (e.g. toluene) in the presence of an acid such as trifluoroacetic acid. The reaction may conveniently be carried out at a temperature of from −20° C. to reflux to provide a compound of formula (II) in one step. The reaction may also be carried out in a solvent such as an aromatic hydrocarbon (e.g. benzene or toluene) under reflux, optionally using a Dean-Stark apparatus to trap the water produced.

The reaction provides a mixture of cis and trans isomers which may be either individual enantiomers or racemates of pairs of cis or trans isomers depending upon whether racemic or enantiomerically pure tryptophan alkyl ester was used as the starting material. Individual cis or trans enantiomers may conveniently be separated from mixtures thereof by fractional crystallisation or by chromatography (e.g. flash column chromatography) using appropriate solvents and eluents. Similarly, pairs of cis and trans isomers may be separated by chromatography (e.g. flash column chromatography) using appropriate eluents. An optically pure trans isomer may also be converted to an optically pure cis isomer using suitable epimerisation procedures. One such procedure comprises treating the trans isomer or a mixture (e.g. 1:1 mixture) of cis and trans isomers with methanolic or aqueous hydrogen chloride at a temperature of from 0° C. to the refluxing temperature of the solution. The mixture may then be subjected to chromatography (e.g. flash column chromatography) to separate the resulting diastereoisomers, or in the procedure utilising aqueous hydrogen chloride the desired cis isomer precipitates out as the hydrochloride salt which may then be isolated by filtration.

Procedure (b)

This comprises a four-step procedure from a compound of formula (III) or a salt thereof (e.g. the hydrochloride salt). The procedure is particularly suitable for preparing a 1R, 3R isomer of formula (III) from a D-tryptophan alkyl ester of formula (IV) or a salt thereof (e.g. the hydrochloride salt). Thus, a first step (i) comprises treating a compound of formula (IV) with an acid halide R^2COHal (where Hal is as previously defined) in the presence of a base, e.g. an organic base such as a trialkylamine (for example triethylamine), to provide a compound of formula (IV)

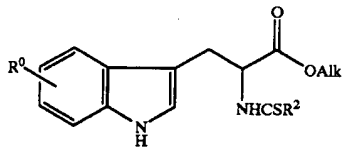
(IV)



The reaction may be conveniently carried out in a suitable solvent such as a halogenated hydrocarbon (e.g. dichloromethane) or an ether (e.g. tetrahydrofuran) and at a temperature of from −20° C. to +40° C.

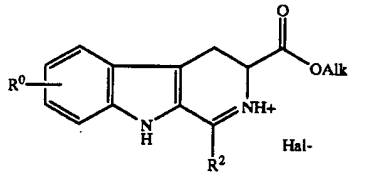
Step (ii) comprises treating a compound of formula (IV) with an agent to convert the amide group to a thioamide group. Suitable sulphurating agents are well-known in the art. Thus, for example, the reaction may conveniently be effected by treating (IV) with Lawesson's reagent. This reaction may conveniently be carried out in a suitable solvent such as an ether (e.g. dimethoxyethane) or an aromatic hydrocarbon (e.g. toluene) at an elevated temperature such as from 40° C. to 80° C. to provide a compound of formula (V)

(V)



Step (iii) comprises treating a compound of formula (V) with a suitable agent to provide a compound of formula (VI)

(VI)

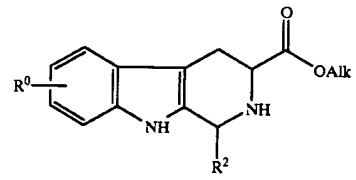


(where Hal is a halogen atom, e.g. iodine). The reaction may conveniently be effected by treating (VI) with an alkylating agent such as a methyl halide (e.g. methyl iodide) or an acylating agent such as an acetyl halide (e.g. acetyl chloride) in a suitable solvent such as a halogenated hydrocarbon (e.g. dichloromethane) at an elevated temperature (e.g. under reflux).

In step (iv) the resulting iminium halide of formula (VI) may be treated with a reducing agent such as boron hydride, e.g. sodium borohydride, to provide the desired compound of formula (II). The reduction may conveniently be effected at a low temperature, e.g. within the range of −100° C. to 0° C., in a suitable solvent such as an alcohol (e.g. methanol).

According to a second process (B), a compound of formula (I) may be prepared by reaction of a compound of formula (VII)

(VII)



where Alk is as previously defined, with the imidazolide of $R^1—NH_2$ under suitable conditions. Compounds of formula (VII) are known in the art and may be made by standard methods.

According to a third process (C), a compound of formula (I) where R^1 represents hydrogen may be prepared by reacting a compound of formula (VII) with urea at elevated temperature.

The pharmaceutically acceptable acid addition salts of the compounds of formula (I) which contain a basic centre may be prepared in a conventional manner. For example, a solution of the free base may be treated with a suitable acid, either neat or in a suitable solution, and the resulting salt isolated either by filtration or by evaporation under vacuum of the reaction solvent. Pharmaceutically acceptable base addition salts may be obtained in an analogous manner by treating a solution of a compound of formula (I) with a suitable base. Both types of salt may be formed or interconverted using ion-exchange resin techniques.

Compounds of the invention may be isolated in association with solvent molecules by crystallisation from or evaporation of an appropriate solvent.

Thus, according to a further aspect of the invention, we provide a process (D) for preparing a compound of formula (I) or a salt or solvate (e.g. hydrate) thereof which comprises process (A) as hereinbefore described followed by

i) an interconversion step; and/or either

ii) salt formation; or

iii) solvate (e.g. hydrate) formation.

The synthesis of the compounds of the invention and of the intermediates for use therein are illustrated by the following, non-limiting Examples.

INTERMEDIATES 1 and 2

Methyl 1,2,3,4-tetrahydro-1-(3,4-methylenedioxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

To a stirred solution of racemic tryptophan methyl ester (13 g) and piperonal (9.7 g) in anhydrous CH_2Cl_2 (300 mL) cooled at 0° C. was added dropwise trifluoroacetic acid (9 mL) and the solution was allowed to react at ambient temperature. After 4 days, the yellow solution was diluted with CH_2Cl_2 (100 mL), washed with a saturated aqueous solution of $NaHCO_3$, then with water and dried over Na_2SO_4. The organic layer was evaporated to dryness under reduced pressure and the residue was purified by flash chromatography eluting with CH_2Cl_2/MeOH (99/1) to give first Intermediate 1, the cis isomer (6.5 g) m.p. :90–93° C. followed by Intermediate 2, the trans isomer (6.4 g) m.p.:170° C.

The following compounds were obtained in a similar manner:

INTERMEDIATES 3 and 4

Methyl 1,2,3,4-tetrahydro-1-(4-methoxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic tryptophan methyl ester and 4-methoxybenzaldehyde gave Intermediate 3, the cis isomer as white crystals m.p.:142° C. and Intermediate 4, the trans isomer as white crystals m.p.:209–210° C.

INTERMEDIATES 5 and 6

Methyl 1,2,3,4-tetrahydro-1-(2-thienyl)-9H-pyrido [3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic tryptophan methyl ester and 2-thiophenecarboxaldehyde gave Intermediate 5, the cis isomer as a pale yellow solid m.p.:134–137° C. and Intermediate 6, the trans isomer as white crystals m.p.:169° C.

INTERMEDIATE 7

Ethyl 1,2,3,4-tetrahydro-1-(4-dimethylaminophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, mixture of cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic tryptophan ethyl ester and 4-dimethylaminobenzaldehyde gave the title compound as white crystals m.p.:170° C.

INTERMEDIATES 8 and 9

Methyl 1,2,3,4-tetrahydro4-fluoro-1-(4-methoxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic 5-fluorotryptophan methyl ester and 4-methoxybenzaldehyde gave Intermediate 8, the cis isomer as a solid 1H NMR ($CDCl_3$) δ(ppm):7.4-6.8(m, 8 H);5.15(brs, 1 H);3.9(dd, 1 H)3.8(s, 3 H);3.2-2.9(m, 2 H) and Intermediate 9, the trans isomer as a solid m.p.:197° C.

INTERMEDIATES 10 and 11

Methyl 1,2,3,4-tetrahydro-1-(4-chlorophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic tryptophan methyl ester and 4-chlorobenzaldehyde gave Intermediate 10, the cis isomer as white crystals m.p.:208–209° C. and Intermediate 11, the trans isomer as white crystals m.p.:108–109° C.

INTERMEDIATES 12 and 13

Methyl 1,2,3,4-tetrahydro-1-(4-trifluoromethylphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method but starting from racemic tryptophan methyl ester and 4-trifluoromethylbenzaldehyde gave Intermediate 12, the cis isomer as pale yellow crystals m.p.:190° C. and Intermediate 13, the trans isomer as pale yellow crystals m.p.:203° C.

INTERMEDIATES 14 and 15

Ethyl 1,2,3,4-tetrahydro-1-(4-cyanophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method but starting from racemic tryptophan ethyl ester and 4-cyanobenzaldehyde gave Intermediate 14, the cis isomer as white crystals m.p.:200° C. and Intermediate 15, the trans isomer as white crystals m.p.: 156° C.

INTERMEDIATES 16 and 17

Ethyl 1,2,3,4-tetrahydro-1-(4-nitrophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method but starting from racemic tryptophan ethyl ester and 4-nitrobenzaldehyde gave Intermediate 16, the cis isomer as yellow crystals m.p.:168° C. and Intermediate 17, the trans isomer as yellow crystals m.p.: 195° C.

INTERMEDIATES 18 and 19

Ethyl 1,2,3,4-tetrahydro-1-(3-pyridyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method but starting from racemic tryptophan ethyl ester and 3-pyridinecarboxaldehyde gave Intermediate 18, the cis isomer as pale yellow crystals m.p.:230–232° C. and Intermediate 19, the trans isomer as white crystals m.p.:210–214° C.

INTERMEDIATES 20 and 21

Ethyl 1,2,3,4-tetrahydro-1-(3-thienyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of Intermediates 1 and 2 but starting from racemic tryptophan ethyl ester and 3-thiophenecarboxaldehyde gave Intermediate 20, the cis isomer as white crystals m.p.:130° C. and Intermediate 21, the trans isomer as white crystals m.p.:182–184° C.

INTERMEDIATE 22

Methyl 1,2,3,4-tetrahydro-1-(3-furyl)-9H-pyrido[3,4-b]indole-3-carboxylate, mixture of cis and trans isomers

The same method but starting from racemic tryptophan methyl ester and 3-furaldehyde gave the title compound as a yellow solid m.p.:130° C.

INTERMEDIATES 23 and 24

(1 R,3 R)-Methyl 1,2,3,4-tetrahydro-1-(3,4-methylenedioxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis isomer and (1 S,3 R)-methyl 1,2,3,4-tetrahydro-1-(3,4-methylenedioxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate trans isomer

To a stirred solution of D-tryptophan methyl ester (11 g) and piperonal (7.9 g) in anhydrous CH_2Cl_2 (400 mL) cooled at 0° C. was added dropwise trifluoroacetic acid (7.7 mL) and the solution was allowed to react at ambient tempera-

ture. After 4 days, the yellow solution was diluted with CH_2Cl_2 (200 mL) and washed with a saturated aqueous solution of $NaHCO_3$, then with water (3x200 mL) and dried over Na_2SO_4. The organic layer was evaporated under reduced pressure and the residue was purified by flash chromatography eluting with dichloromethane/ethyl acetate (97/3) to give first Intermediate 23, the cis isomer (6.5 g) m.p.:154° C. followed by Intermediate 24, the trans isomer (8.4 g) m.p.: 188° C.

INTERMEDIATE 25

Ethyl 1,2,3,4-tetrahydro-6-methyl-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

To a stirred mixture of racemic 5-methyl-tryptophan (4 g) in 1N H_2SO_4 (18 mL) and water (54 mL) was added benzaldehyde (2 mL) and the solution was heated at 80° C. under N_2 for 48 hours.The precipitated product was collected by filtration, washed with water and dried.The crude acid (4.5 g) was then dissolved in ethanol (100 mL) and the solution was cooled at −10° C.Thionyl chloride (1.2 mL) was added dropwise to the solution and the mixture was heated at 60° C. for 48 hours.The solvent was removed under reduced pressure and the residue was taken up in ice water and basified with NH_4OH.The precipited compound was washed with water, dried and purified by flash chromatography eluting with dichloromethane/methanol (98/2) to give first the cis isomer (1.7 g) m.p.:128–130° C., followed by the trans isomer (0.53 g) m.p.:198–200° C.

INTERMEDIATE 26

Ethyl 1,2,3,4-tetrahydro-6-bromo-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same procedure as described in the preparation of Intermediate 25 but starting from racemic 5-bromo-tryptophan and benzaldehyde gave the cis isomer as white crystals m.p.:157–160° C. and the trans isomer as white crystals m.p.:212–216° C.

INTERMEDIATE 27

Methyl 1,2,3,4-tetrahydro-1-(3-chlorophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, mixture of cis and trans isomers

The same method as employed in the preparation of intermediate 1 and 2 but starting from racemic tryptophan methyl ester and 3-chlorobenzaldehyde gave the title compound as white solid m.p.:150–160° C.

INTERMEDIATE 28

Methyl 1,2,3,4-tetrahydro-1-(4-fluorophenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, cis and trans isomers

The same method as employed in the preparation of intermediate 1 and 2 but starting from racemic tryptophan methyl ester and 4-fluorobenzaldehyde gave the cis isomer as white crystals m.p.:92° C. and the trans isomer as pale yellow crystals m.p.:183° C.

INTERMEDIATE 29

Methyl 1,2,3,4-tetrahydro-1-(4-hydroxyphenyl)-9H-pyrido[3,4-b]indole-3-carboxylate, trans isomer

To a stirred solution of racemic tryptophan methyl ester (3 g) and 4-hydroxybenzaldehyde (1.84 g) in anhydrous dichloromethane (50 mL) cooled at 0° C. was added dropwise trifluoroacetic acid (1.27 mL) and the solution was allowed to react at ambient temperature. After 22 hours, the solution was washed with a saturated solution of $NaHCO_3$, then with water, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography eluting with ethyl acetate to give the title compound (3.48 g) as an off-white solid m.p.:233–235° C.

EXAMPLE 1

Cis-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione and Trans-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

To a stirred solution of a mixture of cis and trans isomers of Intermediates 1 and 2 (1 g, 2.85 mmol) in 2-butanone (50 mL) was added dropwise benzyl isocyanate (0.37 mL, 2.99 mmol) and the mixture was refluxed for 15 hours. The solvent was then removed under reduced pressure and the residue was purified by flash chromatography eluting with toluene/ethyl acetate : 85/15 to give first, the trans isomer (240 mg) as white crystals after recrystallisation from diethyl ether. m.p.:208–210° C.

Analysis for $C_{27}H_{21}N_3O_4$:

Calculated: C,71.83;H,4.69;N,9.31;

Found:C,71.46;H,4.77;N,9.24%.

and followed by the cis isomer (470 mg) as white crystals after recrystallisation from ethanol. m.p.:159–161° C.

Analysis for $C_{27}H_{21}N_3O_4$:

Calculated: C,71.83;H,4.69;N,9.31;

Found:C,71.79;H,4.80;N,9.09%.

EXAMPLE 2

Cis-5-(4-methoxyphenyl)-2-methyl-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 3 and methyl isocyanate gave after recrystallisation from ethanol, the title compound as white crystals m.p.:233–240° C.

Analysis for $C_{21}H_{19}N_3O_3$:

Calculated: C,69.79;H,5.30;N,11.63;

Found:C,69.63;H,5.29;N,11.68%.

EXAMPLE 3

Cis-2-ethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione and

Trans-2ethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of Intermediates 3 and 4 and ethyl isocyanate gave the cis isomer as white crystals after recrystallisation from ethanol m.p.:210–220° C.

Analysis for $C_{22}H_{21}N_3O_3$:

Calculated: C,70.38;H,5.64;N,11.19;

Found:C,69.97;H,5.71 ;N,10.83%.

and the trans isomer as white crystals after recrystallisation from 2-propanol m.p.:245–248° C.

Analysis for $C_{22}H_{21}N_3O_3$:

Calculated: C,70.38;H,5.64;N,11.19;

Found:C,70.28;H,5.76;N,11.22%.

EXAMPLE 4

Trans-2-ethyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the Intermediate 2 and ethyl isocyanate gave after recrystallisation from ethyl acetate/hexane, the title compound as white crystals m.p.:238° C.

Analysis for $C_{22}H_{19}N_3O_4$:

Calculated: C,67.86;H,4.92;N,10.79;

Found:C,68.32;H,4.90;N,10.90%.

EXAMPLE 5

Trans-2-ethyl-5-(2-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 6 and ethyl isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:242–248° C.

Analysis for $C_{19}H_{17}N_3O_2S$:

Calculated: C,64.94;H,4.88;N,11.96;

Found:C,64.79;H,5.00;N,11.88%.

EXAMPLE 6

Trans-5-(4-dimethylaminophenyl)-2-ethyl-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1.6] pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of cis and trans isomers of Intermediate 7 and ethyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:262–265° C.

Analysis for $C_{23}H_{24}N_4O_2$:

Calculated: C,71.11;H,6.23;N,14.42;

Found:C,71.01;H,6.29;N,14.49%.

EXAMPLE 7

Trans-2-butyl-9-methyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the trans isomer of Intermediate 25 and butyl isocyanate gave after recrystallisation from diisopropyl ether, the title compound as white crystals m.p. :196–198° C.

Analysis for $C_{24}H_{25}N_3O_2$:

Calculated: C,74.39;H,6.50;N,10.84;

Found:C,74.38;H,6.52;N,10.63%.

EXAMPLE 8

Trans-9-bromo-2-butyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the trans isomer of Intermediate 26 and butyl isocyanate gave after recrystallisation from diisopropyl ether, the title compound as white crystals m.p. :207–210° C.

Analysis for $C_{23}H_{22}BrN_3O_2$:

Calculated: C,61.07;H,4.90;Br,17.66;N,9.29;

Found:C,61.28;H,4.95;Br,17.53;N,9.10%.

EXAMPLE 9

Cis-2-butyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the Intermediate 3 and butyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:220–225° C.

Analysis for $C_{24}H_{25}N_3O_3$:

Calculated: C,71.44;H,6.25;N,10.41;

Found:C,71.56;H,6.23;N,10.36%.

EXAMPLE 10

Trans-2-butyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the Intermediate 4 and butyl isocyanate gave after recrystallisation from ethanol/water, the title compound as white crystals m.p.:173–174° C.

Analysis for $C_{24}H_{25}N_3O_3$:

Calculated: C,71.44;H,6.25;N,10.41;

Found:C,71.53;H,6.20;N,10.28%.

EXAMPLE 11

Cis-2-butyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 8 and butyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:125–130° C.

Analysis for $C_{24}H_{24}FN_3O_3$ ($0.3H_2O$)

Calculated: C,67.53;H,5.81;N,9.84;

Found:C,67.19;H,5.74,N,9.85%.

EXAMPLE 12

Trans-2-butyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the Intermediate 9 and butyl isocyanate gave after recrystallisation from diisopropyl ether/pentane, the title compound as white crystals m.p.:187–189° C.

Analysis for $C_{24}H_{24}FN_3O_3$:

Calculated: C,68.39;H,5.74;N,9.97;

Found:C,68.61;H,5.71;N,10.04%.

EXAMPLE 13

Trans-2-butyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 2 and butyl

isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:152° C.

Analysis for $C_{24}H_{23}N_3O_4$:

Calculated: C,69.05;H,5.55;N,10.07;

Found:C,68.93;H,5.49;N,9.99%.

EXAMPLE 14

Cis-2-butyl-5-(3-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione and Trans-2-butyl-5-(3-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1', 5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of cis and trans isomers of Intermediate 27 and butyl isocyanate gave the cis isomer as pale yellow crystals after recrystallisation from diethyl ether/cyclohexane m.p.:215–217° C.

Analysis for $C_{23}H_{22}ClN_3O_2$:

Calculated: C,67.73;H,5.44;Cl,8.69;N,10.30;

Found:C,67.62;H,5.49;Cl,8.59;N,10.03%.

and the trans isomer as white crystals after recrystallisation from ethanol m.p.: 207–209° C.

Analysis for $C_{23}H_{22}ClN_3O_2$:

Calculated: C,67.73;H,5.44;Cl,8.69;N,10.30;

Found:C,67.60;H,5.41 ;Cl,8.77;N,10.20%.

EXAMPLE 15

Cis-2-butyl-5-(4-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 10 and butyl isocyanate gave after recrystallisation from methanol, the title compound as pale yellow crystals m.p.:252° C.

Analysis for $C_{23}H_{22}ClN_3O_2$:

Calculated: C,67.73;H,5.44;Cl,8.69;N,10.30;

Found:C,67.60;H,5.44;Cl,8.55;N,10.30%.

EXAMPLE 16

Trans-2-butyl-5-(4-chlorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 11 and butyl isocyanate gave after recrystallisation from methanol, the title compound as pale yellow crystals m.p.:174° C.

Analysis for $C_{23}H_{22}ClN_3O_2$:

Calculated: C,67.73;H,5.44;Cl,8.69;N,10.30;

Found:C,67.75;H,5.49;Cl,8.75;N,10.46.%

EXAMPLE 17

Trans-2-butyl-5-(4-fluorophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from the trans isomer of Intermediate 28 and butyl isocyanate gave after recrystallisation from 2-propanol, the title compound as pale yellow crystals m.p. :242° C.

Analysis for $C_{23}H_{22}FN_3O_2$:

Calculated: C,70.57;H,5.66;F,4.85;N,10.73;

Found:C,70.57;H,5.63;F,4.66;N,10.83%.

EXAMPLE 18

Trans-2-butyl-5-(4-hydroxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 29 and butyl isocyanate gave after recrystallisation from 2-propanol/water, the title compound as white crystals m.p.:259° C.

Analysis for $C_{23}H_{23}N_3O_3$:

Calculated: C,70.93;H,5.95;N,10.79;

Found:C,70.41 ;H,6.04;N, 10.63%.

EXAMPLE 19

Cis-2-butyl-5-(4-trifluoromethylphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 12 and butyl isocyanate gave after recrystallisation from methanol/water, the title compound as pale yellow crystals m.p.:232° C.

Analysis for $C_{24}H_{22}F_3N_3O_2$:

Calculated: C,65.30;H,5.02;F,12.91;N,9.52;

Found:C,65.29;H,5.05;F,12.56;N,9.37%.

EXAMPLE 20

Cis-2-butyl-5-(4-cyanophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b] indole-1,3(2H)-dione

The same method as used in the preparation of Example 1 but starting from Intermediate 14 and butyl isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:260° C.

Analysis for $C_{24}H_{22}N_4O_2$:

Calculated: C,72.34;H,5.57;N,14.06;

Found:C,72.30;H,5.59;N,14.08%.

EXAMPLE 21

Trans-2-butyl-5-(4-cyanophenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 15 and butyl isocyanate gave after recrystallisation from diethyl ether/cyclohexane, the title compound as white crystals m.p.:158° C.

Analysis for $C_{24}H_{22}N_4O_2$:

Calculated: C,72.34;H,5.57;N,14.06;

Found:C,72.40;H,5.56;N,13.95%.

EXAMPLE 22

Cis-2-butyl-5-(4-nitrophenyl)-5,6,11,11a-tetrahydro-1H-imidazc[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione and Trans-2-butyl-5-(4-nitrophenyl)-5,6,11, 11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of Intermediates 16

and 17 and butyl isocyanate gave the cis isomer as yellow crystals after recrystallisation from methanol m.p.:236° C.

Analysis for $C_{23}H_{22}N_4O_4$:

Calculated: C,66.02;H,5.30;N,13.39;

Found:C,65.82;H,5.36;N,13.25%.

and the trans isomer as yellow crystals after recrystallisation from 2-propanol m.p.:206° C.

Analysis for $C_{23}H_{22}N_4O_4$:

Calculated: C,66.02;H,5.30;N,13.39;

Found:C,66.12;H,5.38;N,13.28%.

EXAMPLE 23

Cis-2-butyl-5-(3-pyridyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 18 and butyl isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:257–263° C.

Analysis for $C_{22}H_{22}N_4O_2$:

Calculated: C,70.57;H,5.92;N,14.96;

Found:C,70.38;H,6.07;N,14.88%.

EXAMPLE 24

Cis-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione and Trans-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of Intermediates 20 and 21 and butyl isocyanate gave the cis isomer as white crystals after recrystallisation from 2-propanol m.p.:219–221° C.

Analysis for $C_{21}H_{21}N_3O_2S$:

Calculated: C,66.47;H,5.58;N,11.07;S,8.45;

Found:C,66.13;H,5.68;N,11.00;S,8.27%.

and the trans isomer as white crystals after recrystallisation from ethyl acetate m.p.:240–242° C.

Analysis for $C_{21}H_{21}N_3O_2S$:

Calculated: C,66.47;H,5.58;N,11.07;S,8.45;

Found:C,66.68;H,5.69;N,11.05;S,8.56%.

EXAMPLE 25

Cis-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione and Trans-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione

The same method but starting from a mixture of cis and trans isomers Intermediate 22 and butyl isocyanate gave the cis isomer as white crystals after recrystallisation from toluene m.p.:155–160° C.

Analysis for $C_{21}H_{21}N_3O_3$:

Calculated: C,69.41;H,5.82;N, 11.56;

Found:C,69.44;H,5.86;N,11.52%.

and the trans isomer as pale yellow crystals after recrystallisation from ethanol m.p.:215–219° C.

Analysis for $C_2H_{21}N_3O_3$:

Calculated: C,69.41;H,5.82;N,11.56;

Found:C,69.43;H,5.73;N,11.46%.

EXAMPLE 26

Cis-2-cyclohexyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b] indole-1,3(2H)-dione and Trans-2-cyclohexyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from a mixture of Intermediates 3 and 4 and cyclohexyl isocyanate gave the cis isomer as white crystals after recrystallisation from ethanol m.p.:250–260° C.

Analysis for $C_{26}H_{27}N_3O_3$:

Calculated: C,72.71;H,6.34;N,9.78;

Found:C,72.73;H,6.39;N,9.63%.

and the trans isomer as white crystals after recrystallisation from 2-propanol m.p.:265–269° C.

Analysis for $C_{26}H_{27}N_3O_3$:

Calculated: C,72.71;H,6.34;N,9.78;

Found:C,72.82;H,6.38;N,9.69%.

EXAMPLE 27

Cis-2-cyclohexyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 8 and cyclohexyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:275–278° C.

Analysis for $C_{26}H_{26}FN_3O_3$:

Calculated: C,69.78;H,5.86;N,9.39;

Found:C,69.75;H,5.85;N,8.96%.

EXAMPLE 28

Trans-2-cyclohexyl-9-fluoro-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 9 and cyclohexyl isocyanate gave after recrystallisation from ethanol, the title compound as white crystals m.p.:265–267° C.

Analysis for $C_{26}H_{26}FN_3O_3$:

Calculated: C,69.78;H,5.86;N,9.39;

Found:C,69.71;H,5.91;N,9.37%.

EXAMPLE 29

Trans-2-benzyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate[1] and benzyl isocyanate gave after recrystallisation from diethyl ether, the title compound as white crystals m.p.:200–202° C.

Analysis for $C_{26}H_{21}N_3O_2$:

Calculated: C,76.64;H,5.19;N,10.31;

Found:C,76.75;H,5.18;N,10.23%.

1. Cook J., Sandrin J. and Soerens D., Heterocycles, 4, no. 7, 1249–1255 (1976).

EXAMPLE 30

Cis-2-benzyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 3 and benzyl isocyanate gave after recrystallisation from ethanol, the title compound as pale yellow crystals m.p.:240–243° C.

Analysis for $C_{27}H_{23}N_3O_3$:

Calculated: C,74.13;H,5.30;N,9.60;

Found:C,74.13;H,5.31 ;N,9.58%.

EXAMPLE 31

Trans-2-benzyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 4 and benzyl isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:208–212° C.

Analysis for $C_{27}H_{23}N_3O_3$:

Calculated: C,74.13;H,5.30;N,9.60;

Found:C,74.25;H,5.47;N,9.49%.

EXAMPLE 32

(5R,11aR)-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 23 and benzyl isocyanate, gave after recrystallisation toluene, the title compound as white crystals m.p.:145° C.

Analysis for $C_{27}H_{21}N_3O_4$:

Calculated: C,71.83;H,4.69;N,9.31;

Found:C,71.47;H,4.74;N,9.28%.

EXAMPLE 33

Trans-2-benzyl-5-(4-hydroxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 29 and benzyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:268–272° C.

Analysis for $C_{26}H_{21}N_3O_3$:

Calculated: C,73.74;H,5.00;N,9.92;

Found:C,73.63;H,5.09;N,10.02%.

EXAMPLE 34

Trans-2-(2-chloroethyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 4 and 2-chloroethyl isocyanate, gave after recrystallisation from diethyl ether/hexane, the title compound as white crystals m.p.:218–219° C.

Analysis for $C_{22}H_{20}ClN_3O_3$:

Calculated: C,64.47;H,4.92;Cl,8.65;N,10.25;

Found:C,64.44;H,4.98;Cl,8.81;N,10.20%.

EXAMPLE 35

Cis-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from cis methyl 1,2,3,4-tetrahydro-1-cyclohexyl-9H-pyrido[3,4-b]indole-3-carboxylate[1] and benzyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:170–173° C.

Analysis for C26H27N3O2:

Calculated: C,75.52;H,6.58;N,10.16;

Found:C,75.63;H,6.48;N,9.75%.

1. Cook J., Sandrin J. and Soerens D., Heterocycles, 4, no 7, 1249–1255 (1976).

EXAMPLE 36

Trans-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from trans methyl 1,2,3,4-tetrahydro-1-cyclohexyl-9H-pyrido[3,4-b]indole-3-carboxylate[1] and benzyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:130–135° C.

Analysis for C26H27N3O2:

Calculated: C,75.52;H,6.58;N,10.16;

Found:C,75.74;H,6.67;N,9.94%.

1 . Cook J., Sandrin J. and Soerens D., Heterocycles, 4, no 7, 1249–1255 (1976).

EXAMPLE 37

Trans-2-butyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and butyl isocyanate gave after recrystallisation from 2-propanol, the title compound as white crystals m.p.:240–243° C.

Analysis for C23H23N3O2:

Calculated: C,73.97;H,6.21;N,11.25;

Found:C,73.95;H,6.32;N,11.28%.

EXAMPLE 38

Trans-2-cyclohexyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and cyclohexyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:248–250° C.

Analysis for C25H25N3O2:

Calculated: C,75.16;H,6.31 ;N,10.52;

Found:C,75.23;H,6.33;N,10.60%.

EXAMPLE 39

Cis-2-cyclohexyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from cis methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and cyclohexyl isocyanate gave after recrystallisation from methanol, the title compound as white crystals m.p.:267–270° C.

Analysis for C25H25N3O2:

Calculated: C,75.16;H,6.31;N,10.52;

Found:C,75.20;H,6.33;N,10.52%.

EXAMPLE 40

Trans-2-ethoxycarbonylmethyl-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 1 but starting from Intermediate 4 and ethyl isocyanatoacetate gave after recrystallisation from ethanol, the title compound as white crystals m.p.:165–167° C.

Analysis for C24H23N3O5:

Calculated: C,66.50;H,5.35;N,9.69;

Found:C,66.66;H,5.32;N,9.66%.

EXAMPLE 41

Trans-5-(4-methoxyphenyl)-2-[2-(2-pyridyl)-ethyl]-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

To a stirred solution of carbonyl diimidazole (0.28 g, 1.72 mmol) in dry tetrahydrofuran (5 mL), was added dropwise a solution of 2-(2-aminoethyl) pyridine (0.205 g, 1.68 mmol) in tetrahydrofuran (3 mL) and the solution was stirred at room temperature for 0.5 hour. Then, a solution of Intermediate 4 (0.5 g, 1.43 mmol) in dry tetrahydrofuran (7 mL) was added and the resulting solution was refluxed for 20 hours. The solvant was removed under reduced pressure and the residue was dissolved in dichloromethane (50 mL). The solution was washed three times with water (3x20 mL), dried over Na2SO4 and concentrated. The residue was then purified by flash chromatography eluting with dichloromethane/methanol : 99/1 and recrystallised from ethanol/water to give the title compound (0.35 g) as white crystals m.p.:140–143° C.

Analysis for C27H24N4O3:

Calculated: C,71.67;H,5.35;N,12.38;

Found:C,71.87;H,5.41 ;N,12.28%.

EXAMPLE 42

Trans-2-cyclopropyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and cyclopropylamine gave after recrystallisation from ethanol, the title compound as white crystals m.p.:250–255° C.

Analysis for C22H19N3O2:

Calculated: C,73.93;H,5.36;N,11.76;

Found:C,73.84;H,5.45;N,11.63%.

EXAMPLE 43

Trans -2-phenethyl-5-phenyl-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and phenethylamine gave after recrystallisation from diethyl ether, the title compound as white crystals m.p.:240–242° C.

Analysis for C27H23N3O2:

Calculated: C,76.94;H,5.50;N,9.97;

Found:C,77.20;H,5.65;N,10.05%.

EXAMPLE 44

Trans-5-phenyl-2-(2-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and 2-(aminomethyl) pyridine, gave after recrystallisation from methanol, the title compound as white crystals m.p.:165–175° C.

Analysis for C25H20N4O2:

Calculated: C,73.51;H,4.94;N,13.72;

Found:C,73.46;H5.29;N,13.84%.

EXAMPLE 45

Trans-5-phenyl-2-(4-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and 4-(aminomethyl) pyridine, gave after recrystallisation from methanol, the title compound as white crystals m.p.:247–249° C.

Analysis for C25H20N4O2:

Calculated: C,73.51;H,4.94;N,13.72;

Found:C,73,41;H,4.98;N,13.62%.

EXAMPLE 46

Trans-5-(4-methoxyphenyl)-2-(3-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and 3-(aminomethyl) pyridine, gave after recrystallisation from ethanol, the title compound as white crystals m.p.:160–165° C.

Analysis for C26H22N4O3:

Calculated: C,71.22;H,5.06;N,12.78;

Found:C,71.12;H,5.15;N,12.59%.

EXAMPLE 47

Trans-2-(2-dimethylamino-ethyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and N,N-

dimethyl-ethane-1,2-diamine, gave after recrystallisation from ethanol/water, the title compound as pale yellow crystals m.p.:120–124° C.

Analysis for C24H26N4O3:

Calculated: C,68.88;H,6.26;N,13.39;

Found:C,68.91;H,6.43.N,13.23%.

EXAMPLE 48

Trans-2-(3-dimethylamino-propyl)-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and N,N-dimethyl-propane-1,3-diamine, gave after recrystallisation from ethyl acetate/hexane, the title compound as white crystals m.p.:159–161° C.

Analysis for C25H28N4O3:

Calculated: C,69.42;H,6.53;N,12.95;

Found:C,68.89;H,6.60;N,12.91%.

EXAMPLE 49

Trans-2-(2-morpholin-4-yl-ethyl)-5-phenyl-5,6,11, 11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b] indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from trans methyl 1,2,3,4-tetrahydro-1-phenyl-9H-pyrido[3,4-b]indole-3-carboxylate and 2-morpholin-4-yl-ethylamine, gave after recrystallisation from ethanol, the title compound as white crystals m.p.:183–185° C.

Analysis for C25H26N4O3:

Calculated: C,69.75;H,6.09;N,13.01;

Found:C,69.68;H,6.17;N,12.80%.

EXAMPLE 50

Trans-5-(4-methoxyphenyl)-2-[3-(4-methyl-piperazin-1-yl)-propyl]- 5,6,11,11-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and 3-(4-methyl-piperazin-1-yl)-propylamine, gave after recrystallisation from ethanol/water, the title compound as white crystals m.p.: 164–168° C.

Analysis for C28H33N5O3 (0.5 H2O):

Calculated: C,67.72;H,6.9;N,14.1;

Found:C,67.85;H,6.75;N,14.13%.

EXAMPLE 51

Trans-5-(4-methoxyphenyl)-2-(2-pyrrolidin-1-yl-ethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and 2-pyrrolidin-1-yl-ethylamine, gave after recrystallisation from ethanol/water, the title compound as white crystals m.p.:126–130° C.

Analysis for C26H28N4O3:

Calculated: C,70.25;H,6.35;N,12.00;

Found:C,69.99;H,6.35;N,12.50%.

EXAMPLE 52

Trans-5-(4-methoxyphenyl)-2-[2-(1-methyl-pyrrolidin-2-yl)-ethyl]-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6] pyrido [3,4-b]indole-1,3(2H)-dione

The same method as employed in the preparation of Example 41 but starting from Intermediate 4 and 2-(1-methyl-pyrrolidin-2-yl)-ethylamine, gave after recrystallisation from methanol, the title compound as white crystals m.p.:170–180° C.

Analysis for C27H30N4O3:

Calculated: C,70.72;H,6.59;N,12.22;

Found:C,70.86;H,6.62;N,12.41%.

EXAMPLE 53

Trans-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3 (2H)-dione

A mixture of Intermediate 4 (0.5 g, 1.48 mmol) and urea (0.1 g) was heated at 220° C. for a few minutes. The reaction was then cooled to room temperature and the solid suspended in methanol, filtered then recrystallised from hot methanol to give the title compound as off-white crystals m.p.:295–305° C.

Analysis for C20H17N3O3:

Calculated: C,69.15;H,4.93;N,12.10;

Found:C,68.87;H,4.95;N,12.00%.

EXAMPLE 54

Cis-5-(4-methoxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo[1',5':1,6] pyrido [3,4-b]indole-1,3 (2H)-dione

The same method as employed in the preparation of Example 53 but starting from Intermediate 3 and urea, gave after recrystallisation from methanol, the title compound as pale yellow crystals m.p.:300–310° C.

Analysis for C20H17N3O3:

Calculated: C,69.15;H,4.93;N,12.10;

Found:C,68.90;H,4.91;N,11.98%.

TABLETS FOR ORAL ADMINISTRATION

A. Direct Compression

1.	mg/tablet
Active ingredient	50.0
Crospovidone USNF	8.0
Magnesium Stearate Ph Eur	1.0
Anhydrous Lactose	141.0

The active ingredient was sieved and blended with the excipients. The resultant mix was compressed into tablets.

2.	mg/tablet
Active ingredient	50.0
Colloidal Silicon Dioxide	0.5

-continued

2.	mg/tablet
Crospovidone	8.0
Sodium Lauryl Sulphate	1.0
Magnesium Stearate Ph Eur	1.0
Microcrystalline Cellulose USNF	139.5

The active ingredient was sieved and blended with the excipients. The resultant mix was compressed into tablets.

B. Wet Granulation

1.	mg/tablet
Active ingredient	50.0
Polyvinyl pyrollidone	150.0
Polyethylene glycol	50.0
Polysorbate 80	10.0
Magnesium Stearate Ph Eur	2.5
Croscarmellose Sodium	25.0
Colloidal Silicon Dioxide	2.5
Microcrystalline Cellulose USNF	210.0

The polyvinyl pyrollidone, polyethylene glycol and polysorbate 80 were dissolved in water. The resultant solution was used to granulate the active ingredient. After drying the granules were screened, then extruded at elevated temperatures and pressures. The extrudate was milled and/or screened then was blended with the microcrystalline cellulose, croscarmellose sodium, colloidal silicon dioxide and magnesium stearate. The resultant mix was compressed into tablets.

2.	mg/tablet
Active ingredient	50.0
Polysorbate 80	3.0
Lactose Ph Eur	178.0
Starch BP	45.0
Pregelatinised Maize Starch BP	22.5
Magnesium Stearate BP	1.5

The active ingredient was sieved and blended with the lactose, starch and pregelatinised maize starch. The polysorbate 80 was dissolved in purified water. Suitable volumes of the polysorbate 80 solution were added and the powders were granulated. After drying, the granules were screened and blended with the magnesium stearate. The granules were then compressed into tablets.

Tablets of other strengths may be prepared by altering the ratio of active ingredient to the other excipients.

FILM COATED TABLETS

The aforementioned tablet formulations were film coated.

Coating Suspension	% w/w
Opadry white†	13.2
Purified water Ph Eur	to100.0*

The water did not appear in the final product. The maximum theoretical weight of solids applied during coating was 20 mg/tablet.

†Opadry white is a proprietary material obtainable from Colorcon Limited, UK which contains hydroxypropyl methylcellulose, titanium dioxide and triacetin.

The tablets were film coated using the coating suspension in conventional film coating equipment.

Capsules

1.	mg/capsule
Active ingredient	50.0
Lactose	148.5
Polyvinyl pyrollidone	100.0
Magnesium Stearate	1.5

The active ingredient was sieved and blended with the excipients. The mix was filled into size No. 1 hard gelatin capsules using suitable equipment.

2.	mg/capsule
Active ingredient	50.0
Microcrystalline Cellulose	233.5
Sodium Lauryl Sulphate	3.0
Crospovidone	12.0
Magnesium Stearate	1.5

The active ingredient was sieved and blended with the excipients. The mix was filled into size No. 1 hard gelatin capsules using suitable equipment.

Other doses may be prepared by altering the ratio of active ingredient to excipient, the fill weight and if necessary changing the capsule size.

3.	mg/capsule
Active ingredient	50.0
Labrafil M1944CS	to1.0 ml

The active ingredient was sieved and blended with the Labrafil. The suspension was filled into soft gelatin capsules using appropriate equipment.

Inhibitory effect on cGMP-PDE

cGMP-PDE activity of compounds of the present invention was measured using a one-step assay adapted from Wells at al. (Wells, J. N., Baird, C. E., Wu, Y. J. and Hardman, J. G., Biochim. Biophys. Acta 384, 430 (1975)). The reaction medium contained 50 mM Tris-HCl,pH 7.5, 5 mM Mg-acetate, 250μg/ml 5'-Nucleotidase, 1 mM EGTA and 0.15μM 8-[H^3]-cGMP. The enzyme used was a human recombinant PDE V (ICOS, Seattle USA).

Compounds of the invention were dissolved in DMSO finally present at 2% in the assay. The incubation time was 30 minutes during which the total substrate conversion did not exceed 30%.

The IC_{50} values for the compounds examined were determined from concentration-response curves using typically concentrations ranging from 10 nM to 10μM. Tests against other PDE enzymes using standard methodology also showed that compounds of the invention are highly selective for the CGMP specific PDE enzyme.

cGMP level measurements Rat aortic smooth muscle cells (RSMC) prepared according to Chamley et al. in Cell Tissue Res. 177, 503–522 (1977) were used between the 10th and 25th passage at confluence in 24-well culture dishes. Culture media was aspirated and replaced with PBS (0.5 ml) containing the compound tested at the appropriate concentration. After 30 minutes at 37° C., particulates guanylate cyclase was stimulated by addition of ANF (100 nM) for 10 minutes. At the end of incubation, the medium was withdrawn and two extractions were performed by addition of 65% ethanol (0.25 ml). The two ethanolic extracts were pooled and evaporated until dryness, using a Speed-vac

system. c-GMP was measured after acetylation by scintillation proximity immunoassay (AMERSHAM). The EC_{50} values are expressed as the dose giving half of the stimulation at saturating concentrations

BIOLOGICAL DATA

The compounds according to the present invention were typically found to exhibit an IC_{50} value of less than 500 nM and an EC_{50} value of less than 5 μM. In vitro test data for representative compounds of the invention is given in the following table:

TABLE 1

In vitro results

Example No.	IC_{50} nM	EC_{50} μM
10	4	<1
26 (cis isomer)	7	0.3
1 (cis isomer)	<10	0.3
32	<10	0.2

The hypotensive effects of compounds according to the invention as identified in Table 2 were studied in conscious spontaneously hypertensive rats (SHRs). The compounds were administered orally at a dose of 5 or 10 mg/kg in a mixture of 5% DMF and 95% olive oil, or i.v. at a dose of 10 mg/kg in a mixture of 40% dimethylformamide, 25% tetraglycol, and 25% glucose serum. Blood pressure was measured from a catheter inserted in the carotid artery and recorded for 5 hours after administration. The results are expressed as Area Under the Curve (AUC from 0 to 5 hours, mmHg.hour) of the fall in blood pressure over time.

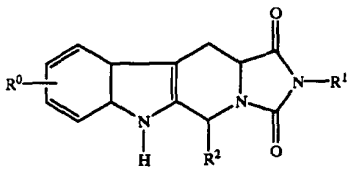
TABLE 2

In vitro results

Example No.	AUC (mmHg.h)
10	147 (dosed at 10 mg/kg i.v.)
26 (cis isomer)	117 (dosed at 10 mg/kg i.v.)
1 (cis isomer)	104 (dosed at 5 mg/kg p.o.)
32	65 (dosed at 5 mg/kg p.o.)

We claim:

1. A compound of formula (I)



(I)

and salts and solvates thereof, in which:

R^0 represents hydrogen, halogen or C_{1-6}alkyl;

R^1 is selected from the group consisting of:

(a) hydrogen;

(b) C_{1-6}alkyl optionally substituted by one or more substituents selected from phenyl, halogen, $—CO_2R^a$ and $—NR^aR^b$;

(c) C_{3-6}cycloalkyl;

(d) phenyl; and

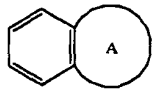
(e) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur, and being optionally substituted by one or more C_{1-6}alkyl, and optionally linked to the nitrogen atom to which R^1 is attached via C_{1-6}alkyl;

R^2 is selected from the group consisting of:

(f) C_{3-6}cycloalkyl;

(g) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen and sulphur; and

(h) a bicyclic ring



attached to the rest of the molecule via one of the benzene ring carbon atoms and A is a 5- or 6-membered heterocyclic ring as defined in point (g); and

R^a and R^b independently represent hydrogen or C_{1-6}alkyl.

2. A compound according to claim 1 wherein R^0 is selected from hydrogen, methyl, bromine and fluorine.

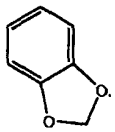
3. A compound according to claim 1 wherein R^1 is selected from methyl, ethyl optionally substituted by one or more chlorine atoms, butyl, cyclohexyl and benzyl.

4. A compound according to claim 1 wherein R^1 is selected from hydrogen, cycloalkyl, C_{1-6}alkyl substituted by an $—NR^aR^b$ substituent, phenyl optionally linked to the nitrogen atom to which R^1 is attached via a C_{1-6}alkyl chain, and C_{1-6}alkyl, substituted by $—CO_2R^a$.

5. A compound according to claim 1 wherein R^1 is selected from pyridyl, morpholinyl, piperazinyl, pyrrolidinyl and piperidinyl, such rings being linked to the nitrogen atom to which R^1 is attached via a C_{1-6}alkyl chain.

6. A compound according to claim 5 wherein the heterocyclic ring is linked to the nitrogen atom to which R^1 is attached via a C_{1-4}alkyl chain.

7. A compound according to any of claims 1 wherein R^2 represents



8. A compound according to claim 1 wherein R^2 represents thienyl, pyridyl, or furyl.

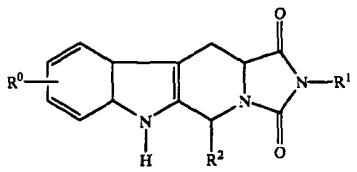
9. A compound according to claim 1 wherein R^2 represents a C_{3-6}cycloalkyl group.

10. The method of claim 1 wherein the condition is erectile dysfunction.

11. The method of claim 10 wherein the animal body is human.

12. The method of claim 10 wherein the compound is administered orally.

13. A method of treating conditions where inhibition of cGMP-specific PDE is of therapeutic benefit, in a human or nonhuman animal body, which comprises administering to said body a therapeutically effective amount of a compound having a formula



and salts and solvates thereof, in which:

R^0 represents hydrogen, halogen, or C_{1-6}alkyl;

R^1 is selected from the group consisting of:

(a) hydrogen;

(b) C_{1-6}alkyl optionally substituted by one or more substituents selected from phenyl, halogen, $—CO_2R^a$ and $—NR^aR^b$;

(c) C_{3-6}cycloalkyl;

(d) phenyl; and

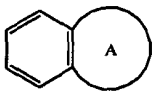
(e) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen, and sulphur, and being optionally substituted by one or more C_{1-6}alkyl, and optionally linked to the nitrogen atom to which R^1 is attached via C_{1-6}alkyl;

R^2 is selected from the group consisting of:

(f) C_{3-6}cycloalkyl;

(g) a 5- or 6-membered heterocyclic ring containing at least one heteroatom selected from oxygen, nitrogen, and sulphur; and

(h) a bicyclic ring



attached to the rest of the molecule via one of the benzene ring carbon atoms and A is a 5- or 6-membered heterocyclic ring as defined in point (h); and R^a and R^b independently represent hydrogen or C_{1-6}alkyl.

14. A compound selected from:

Cis-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3 (2H) -dione;

Trans-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3 (2H) -dione;

Trans-2-ethyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3 (2H) -dione;

Trans-2-ethyl-5-(2-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3 (2H) -dione;

Cis-2-butyl-5-(3-pyridyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b] indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3-thienyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Cis-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido [3,4-b]indole-1,3(2H)-dione;

Trans-2-butyl-5-(3-furyl)-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

(5R,11aR)-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione;

Cis-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-2-benzyl-5-cyclohexyl-5,6,11,11a-tetrahydro-1H-imidazo [1', 5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

Trans-5-phenyl-2-(2-pyridylmethyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3(2H)-dione;

and pharmaceutically acceptable salts and solvates thereof.

15. (5R,11aR)-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b] indole-1,3(2H)-dione; Cis-2-benzyl-5-(3,4-methylenedioxyphenyl)-5,6,11,11a-tetrahydro-1H-imidazo [1',5':1,6]pyrido[3,4-b]indole-1,3-(2H)-dione; and pharmaceutically acceptable salts and solvates thereof.

* * * * *

US006107338A

United States Patent [19]

Wos et al.

[11] Patent Number: 6,107,338

[45] Date of Patent: *Aug. 22, 2000

[54] AROMATIC C_{16}-C_{20}-SUBSTITUTED TETRAHYDRO PROSTAGLANDINS USEFUL AS FP AGONISTS

[75] Inventors: **John August Wos**, Cincinnati; **Mitchell Anthony deLong**, West Chester; **Jack S. Amburgey, Jr.**, Loveland, all of Ohio; **Haiyan George Dai**, Drexel Hill, Pa.; **Cynthia Jean Miley**; **Biswanath De**, both of Cincinnati, Ohio

[73] Assignee: **The Procter & Gamble Company**, Cincinnati, Ohio

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: **09/148,374**

[22] Filed: **Sep. 4, 1998**

Related U.S. Application Data

[60] Provisional application No. 60/058,246, Sep. 9, 1997.

[51] **Int. Cl.**[7] **C07C 405/00**; A61K 31/5575

[52] **U.S. Cl.** **514/530**; 514/570; 514/438; 549/79; 560/60; 562/470; 562/621

[58] **Field of Search** 560/121, 118, 560/60; 562/470, 621; 549/79; 514/530, 570, 438

[56] **References Cited**

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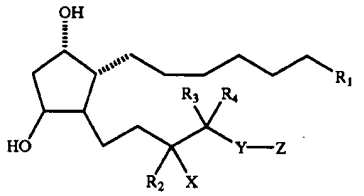
Primary Examiner—Robert Gerstl

Attorney, Agent, or Firm—James C. Kellerman; Carl J. Roof

[57] **ABSTRACT**

The invention provides novel PGF analogs. In particular, the present invention relates to compounds having a structure according to the following formula:



wherein R_1, R_2, R_3, R_4, X, Y, and Z are defined below.

This invention also includes optical isomers, diastereomers and enantiomers of the formula above, and pharmaceutically-acceptable salts, biohydrolyzable amides, esters, and imides thereof.

The compounds of the present invention are useful for the treatment of a variety of diseases and conditions, such as bone disorders and glaucoma. Accordingly, the invention further provides pharmaceutical compositions comprising these compounds. The invention still further provides methods of treatment for bone disorders and glaucoma using theses compounds or the compositions containing them.

26 Claims, No Drawings

AROMATIC C_{16}-C_{20}-SUBSTITUTED TETRAHYDRO PROSTAGLANDINS USEFUL AS FP AGONISTS

CROSS REFERENCE

This application claims priority under Title 35, United States Code 119(e) from Provisional Application Ser. No. 60/058,246, filed Sep. 9, 1997.

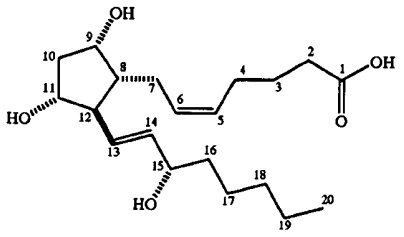
TECHNICAL FIELD

The subject invention relates to certain novel analogs of the naturally occurring prostaglandins. Specifically, the subject invention relates to novel Prostaglandin F analogs. The subject invention further relates to methods of using said novel Prostaglandin F analogs. Preferred uses include methods of treating bone disorders and glaucoma.

BACKGROUND OF THE INVENTION

Naturally occurring prostaglandins (PGA, PGB, PGE, PGF, and PGI) are C-20 unsaturated fatty acids. $PGF_{2\alpha}$, the naturally occurring Prostaglandin F in humans, is characterized by hydroxyl groups at the C_9 and C_{11} positions on the alicyclic ring, a cis-double bond between C_5 and C_6, and a trans-double bond between C_{13} and C_{14}. Thus $PGF_{2\alpha}$ has the following formula:

$PGF_{2\alpha}$



Analogs of naturally occurring Prostaglandin F have been disclosed in the art. For example, see U.S. Pat. No. 4,024,179 issued to Bindra and Johnson on May 17, 1977; German Patent No. DT-002,460,990 issued to Beck, Lerch, Seeger, and Teufel published on Jul. 1, 1976; U.S. Pat. No. 4,128,720 issued to Hayashi, Kori, and Miyake on Dec. 5, 1978; U.S. Pat. No. 4,011,262 issued to Hess, Johnson, Bindra, and Schaaf on Mar. 8, 1977; U.S. Pat. No. 3,776,938 issued to Bergstrom and Sjovall on Dec. 4, 1973; P. W. Collins and S. W. Djuric, "Synthesis of Therapeutically Useful Prostaglandin and Prostacyclin Analogs", *Chem. Rev.* Vol. 93 (1993), pp. 1533–1564; G. L. Bundy and F. H. Lincoln, "Synthesis of 17-Phenyl-18,19,20-Trinorprostaglandins: I. The PG_1 Series", *Prostaglandins*, Vol. 9 No. 1 (1975), pp. 1–4; W. Bartman, G. Beck, U. Lerch, H. Teufel, and B. Scholkens, "Luteolytic Prostaglandins: Synthesis and Biological Activity", *Prostaglandins*, Vol. 17 No. 2 (1979), pp. 301–311; C. Iiljebris, G. Selen, B. Resul, J. Sternschantz, and U. Hacksell, "Derivatives of 17- Phenyl-18,19,20-trinorprostaglandin $F_2\alpha$ Isopropyl Ester: Potential Antiglaucoma Agents", *Journal of Medicinal Chemistry*, Vol. 38 No. 2 (1995), pp. 289–304.

Naturally occurring prostaglandins are known to possess a wide range of pharmacological properties. For example, prostaglandins have been shown to: relax smooth muscle, which results in vasodilatation and bronchodilatation, to inhibit gastric acid secretion, to inhibit platelet aggregation,

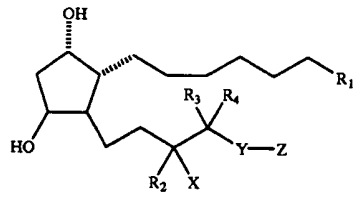
to reduce intraocular pressure, and to induce labor. Although naturally occurring prostaglandins are characterized by their activity against a particular prostaglandin receptor, they generally are not specific for any one prostaglandin receptor. Therefore, naturally-occurring prostaglandins are known to cause side effects such as inflammation, as well as surface irritation when administered systemically. It is generally believed that the rapid metabolism of the naturally occurring prostaglandins following their release in the body limits some of the effects of the prostaglandin to a local area. This effectively prevents the prostaglandin from stimulating prostaglandin receptors throughout the body and causing the effects seen with the systemic administration of naturally occurring prostaglandins.

Prostaglandins, especially prostaglandins of the E series (PGE), are known to be potent stimulators of bone resorption. $PGF_{2\alpha}$ has also been shown to be a stimulator of bone resorption but not as potent as PGE_2. Also, it has been demonstrated the $PGF_{2\alpha}$ has little effect on bone formation. It has been suggested that some of the effects of $PGF_{2\alpha}$ on bone resorption, formation and cell replication may be mediated by an increase in endogenous PGE_2 production.

In view of both the wide range of pharmacological properties of naturally occurring prostaglandins and of the side effects seen with the systemic administration of these naturally occurring prostaglandins, attempts have been made to prepare analogs to the naturally occurring prostaglandins that are selective for a specific receptor or receptors. A number of such analogs have been disclosed in the art. Though a variety of prostaglandin analogs have been disclosed, there is a continuing need for potent, selective prostaglandin analogs for the treatment of a variety diseases and conditions.

SUMMARY OF THE INVENTION

The invention provides novel PGF analogs. In particular, the present invention relates to compounds having a structure according to the following formula:



wherein R_1, R_2, R_3, R_4, X, Y, and Z are defined below.

This invention also includes optical isomers, diastereomers and enantiomers of the formula above, and pharmaceutically-acceptable salts, biohydrolyzable amides, esters, and imides thereof.

The compounds of the present invention are useful for the treatment of a variety of diseases and conditions, such as bone disorders and glaucoma. Accordingly, the invention further provides pharmaceutical compositions comprising these compounds. The invention still further provides methods of treatment for bone disorders and glaucoma using theses compounds or the compositions containing them.

DETAILED DESCRIPTION OF THE INVENTION

Terms and Definitions

"Acyl' is a group suitable for acylating a nitrogen atom to form an amide or carbamate or an oxygen atom to form an

ester group. Preferred acyl groups include benzoyl, acetyl, tert-butyl acetyl, para-phenyl benzoyl, and trifluoroacetyl. More preferred acyl groups include acetyl and benzoyl. The most preferred acyl group is acetyl.

"Alkyl" is a saturated or unsaturated hydrocarbon chain having 1 to 18 carbon atoms, preferably 1 to 12, more preferably 1 to 6, more preferably still 1 to 4 carbon atoms. Alkyl chains may be straight or branched. Preferred branched alkyl have one or two branches, preferably one branch. Preferred alkyl are saturated. Unsaturated alkyl have one or more double bonds and/or one or more triple bonds. Preferred unsaturated alkyl have one or two double bonds or one triple bond, more preferably one double bond. Alkyl chains may be unsubstituted or substituted with from 1 to about 4 substituents. Preferred alkyl are unsubstituted. Preferred substituted alkyl are mono-, di-, or trisubstituted. Preferred alkyl substituents include methyl, ethyl, propyl and butyl, halo, hydroxy, alkoxy (e.g., methoxy, ethoxy, propoxy, butoxy, pentoxy), aryloxy (e.g., phenoxy, chlorophenoxy, tolyloxy, methoxyphenoxy, benzyloxy, alkyloxycarbonylphenoxy, acyloxyphenoxy), acyloxy (e.g., propionyloxy, benzoyloxy, acetoxy), carbamoyloxy, carboxy, mercapto, alkylthio, acylthio, arylthio (e.g., phenylthio, chlorophenylthio, alkylphenylthio, alkoxyphenylthio, benzylthio, alkyloxycarbonylphenylthio), aryl (e.g., phenyl, tolyl, alkyloxphenyl, alkyloxycarbonylphenyl, halophenyl), heterocyclyl, heteroaryl, amino (e.g., amino, mono- and di- C_1–C_3 alkanylamino, methylphenylamino, :methylbenzylamino, C_1–C_3 alkanylamido, carbamamido, ureido, guanidino).

"Aromatic ring" is an aromatic hydrocarbon ring system. Aromatic rings are monocyclic or fused bicyclic ring systems. Monocyclic aromatic rings contain from about 5 to about 10 carbon atoms, preferably from 5 to 7 carbon atoms, and most preferably from 5 to 6 carbon atoms in the ring. Bicyclic aromatic rings contain from 8 to 12 carbon atoms, preferably 9 or 10 carbon atoms in the ring. Aromatic rings may be unsubstituted or substituted with from 1 to about 4 substituents on the ring. Preferred aromatic ring substituents include: halo, cyano, alkyl, heteroalkyl, haloalkyl, phenyl, phenoxy or any combination thereof. More preferred substituents include halo and haloalkyl. Preferred aromatic rings include naphthyl and phenyl. The most preferred aromatic ring is phenyl.

"Bone disorder" means the need for bone repair or replacement. Conditions in which the need for bone repair or replacement may arise include: osteoporosis (including post menopausal osteoporosis, male and female senile osteoporosis and corticosteroid induced osteoporosis), osteoarthritis, Paget's disease, osteomalacia, multiple myeloma and other forms of cancer, prolonged bed rest, chronic disuse of a limb, anorexia, microgravity, exogenous and endogenous gonadal insufficiency, bone fracture, non-union, defect, prosthesis implantation and the like.

"Carbocyclic aliphatic ring" is a saturated or unsaturated hydrocarbon ring. Carbocyclic aliphatic rings are not aromatic. Carbocyclic aliphatic rings are monocyclic, or are fused, spiro, or bridged bicyclic ring systems. Monocyclic carbocyclic aliphatic rings contain from about 4 to about 10 carbon atoms, preferably from 4 to 7 carbon atoms, and most preferably from 5 to 6 carbon atoms in the ring. Bicyclic carbocyclic aliphatic rings contain from 8 to 12 carbon atoms, preferably from 9 to 10 carbon atoms in the ring. Carbocyclic aliphatic rings may be unsubstituted or substituted with from 1 to about 4 substituents on the ring. Preferred carbocyclic aliphatic ring substituents include: halo, cyano, alkyl, heteroalkyl, haloalkyl, phenyl, phenoxy or any combination thereof. More preferred substituents include halo and haloalkyl. Preferred carbocyclic aliphatic rings include cyclopentyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl. More preferred carbocyclic aliphatic rings include cyclohexyl, cycloheptyl, and cyclooctyl. The most preferred carbocyclic aliphatic ring is cycloheptyl.

"Halo" is fluoro, chloro, bromo or iodo. Preferred halo are fluoro, chloro and bromo; more preferred are chloro and fluoro, especially fluoro.

"Haloalkyl" is a straight, branched, or cyclic hydrocarbon substituted with one or more halo substituents. Preferred haloalkyl are C_1–C_{12}; more preferred are C_1–C_6; more preferred still are C_1–C_3. Preferred halo substituents are fluoro and chloro. The most preferred haloalkyl is trifluoromethyl.

"Heteroalkyl" is a saturated or unsaturated chain containing carbon and at least one heteroatom, wherein no two heteroatoms are adjacent. Heteroalkyl chains contain from 1 to 18 member atoms (carbon and heteroatoms) in the chain, preferably 1 to 12, more preferably 1 to 6, more preferably still 1 to 4. Heteroalkyl chains may be straight or branched. Preferred branched heteroalkyl have one or two branches, preferably one branch. Preferred heteroalkyl are saturated. Unsaturated heteroalkyl have one or more double bonds and/or one or more triple bonds. Preferred unsaturated heteroalkyl have one or two double bonds or one triple bond, more preferably one double bond. Heteroalkyl chains may be unsubstituted or substituted with from 1 to about 4 substituents. Preferred heteroalkyl are unsubstituted. Preferred heteroalkyl substituents include methyl, ethyl, propyl and butyl, halo, hydroxy, alkoxy (e.g., methoxy, ethoxy, propoxy, butoxy, pentoxy), aryloxy (e.g., phenoxy, chlorophenoxy, tolyloxy, methoxyphenoxy, benzyloxy, alkyloxycarbonylphenoxy, acyloxyphenoxy), acyloxy (e.g., propionyloxy, benzoyloxy, acetoxy), carbamoyloxy, carboxy, mercapto, alkylthio, acylthio, arylthio (e.g., phenylthio, chlorophenylthio, alkylphenylthio, alkoxyphenylthio, benzylthio, alkyloxycarbonylphenylthio), aryl (e.g., phenyl, tolyl, alkyloxphenyl, alkyloxycarbonylphenyl, halophenyl), heterocyclyl, heteroaryl, amino (e.g., amino, mono- and di- C_1–C_3 alkanylamino, methylphenylamino, methylbenzylamino, C_1–C_3 alkanylamido, carbamamido, ureido, guanidino).

"Heteroatom" is a nitrogen, sulfur, or oxygen atom. Groups containing more than one heteroatom may contain different heteroatoms.

"Heterocyclic aliphatic ring" is a saturated or unsaturated ring containing carbon and from 1 to about 4 heteroatoms in the ring, wherein no two heteroatoms are adjacent in the ring and no carbon in the ring that has a heteroatom attached to it also has a hydroxyl, amino, or thiol group attached to it. Heterocyclic aliphatic rings are not aromatic. Heterocyclic aliphatic rings are monocyclic, or are fused or bridged bicyclic ring systems. Monocyclic heterocyclic aliphatic rings contain from about 4 to about 10 member atoms (carbon and heteroatoms), preferably from 4 to 7, and most preferably from 5 to 6 member atoms in the ring. Bicyclic heterocyclic aliphatic rings contain from 8 to 12 member atoms, preferably 9 or 10 member atoms in the ring. Heterocyclic aliphatic rings may be unsubstituted or substituted with from 1 to about 4 substituents on the ring. Preferred heterocyclic aliphatic ring substituents include: halo, cyano, alkyl, heteroalkyl, haloalkyl, phenyl, phenoxy or any combination thereof. More preferred substituents include halo and haloalkyl. Preferred heterocyclic aliphatic rings include piperzyl, morpholinyl, tetrahydrofuranyl, tetrahydropyranyl and piperdyl.

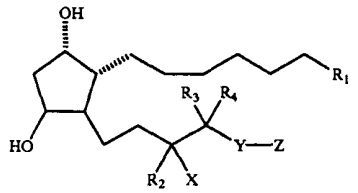
"Heteroaromatic ring" is an aromatic ring system containing carbon and from 1 to about 4 heteroatoms in the ring. Heteroaromatic rings are monocyclic or fused bicyclic ring systems. Monocyclic heteroaromatic rings contain from about 5 to about 10 member atoms (carbon and heteroatoms), preferably from 5 to 7, and most preferably from 5 to 6 member atoms in the ring. Bicyclic heteroaromatic rings contain from 8 to 12 member atoms, preferably 9 or 10 member atoms in the ring. Heteroaromatic rings may be unsubstituted or substituted with from 1 to about 4 substituents on the ring. Preferred heteroaromatic ring substituents include: halo, cyano, alkyl, heteroalkyl, haloalkyl, phenyl, phenoxy or any combination thereof. More preferred substituents include halo, haloalkyl, and phenyl. Preferred heteroaromatic rings include thienyl, thiazolo, purinyl, pyrimidyl, pyridyl, and furanyl. More preferred heteroaromatic rings include thienyl, furanyl, and pyridyl. The most preferred heteroaromatic ring is thienyl.

"Lower alkyl" is an alkyl chain radical comprised of 1 to 6, preferably 1 to 4 carbon atoms.

"Phenyl" is a six-membered monocyclic aromatic ring which may or may not be substituted with from about 1 to about 4 substituents. The substituents may be substituted at the ortho, meta or para position on the phenyl ring, or any combination thereof. Preferred phenyl substituents include: halo, cyano, alkyl, heteroalkyl, haloalkyl, phenyl, phenoxy or any combination thereof. More preferred substituents on the phenyl ring include halo and haloalkyl. The most preferred substituent is halo. The preferred substitution pattern on the phenyl ring is ortho or meta. The most preferred substitution pattern on the phenyl ring is ortho.

Compounds

The subject invention involves compounds having the following structure:



In the above structure, R_1 is CO_2H, C(O)NHOH, CO_2R_5, CH_2OH, $S(O)_2R_5$, $C(O)NHR_5$, $C(O)NHS(O)_2R_5$, or tetrazole; wherein R_5 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring. Preferred R_5 is CH_3, C_2H_5, C_3H_7. Preferred R_1 is CO_2H, C(O)NHOH, CO_2CH_3, $CO_2C_2H_5$, $CO_2C_3H_7$, $CO_2C_4H_9$, $CO_2C_3H_7O_2$, and $C(O)NHS(O)_2R_5$. More preferred R_1 is CO_2H, C(O)NHOH, CO_2CH_3, and $CO_2C_3H_5$. Most preferred R_1 is CO_2H and CO_2CH_3.

In the above structure, R_2 is H or lower alkyl. Preferred R_2 is H and CH_3. Most preferred R_2 is H.

In the above structure, X is NR_6R_7, OR_8, SR_9, $S(O)R_9$, $S(O)_2R_9$, or F; wherein R_6, R_7, and R_8 are independently selected from the group consisting of H, acyl, alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, and heteroaromatic ring; and wherein R_9 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring. Preferred R_6 and R_7 are H, CH_3 and C_2H_5. Preferred R_8 is H, CH_3, C_2H_5, and C_3H_7. Preferred R_9 is CH_3 and C_2H_5. Preferred X is NR_6R_7 and OR_8. Most preferred X is OH.

In the above structure, R_3 and R_4 are independently H, CH_3, C_2H_5, OR_{10}, SR_{10}, or OH, except that both R_3 and R_4 are not OH; wherein R_{10} is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring, R_{10} having from 1 to about 8 member atoms. Preferred R_3 and R_4 are H.

In the above structure, Y is $(CH_2)_n$; n being an integer from 0 to about 3. Preferred n is 0,1, and 2. Most preferred n is 1.

In the above structure, Z is carbocyclic aliphatic ring, heterocyclic aliphatic ring, monocyclic heteroaromatic ring, or substituted phenyl when n is 0, 2, or 3; and Z is carbocyclic aliphatic ring, heterocyclic aliphatic ring, or substituted phenyl when n is 1. Preferred Z is monocyclic. More preferred Z is substituted phenyl and monocyclic heteroaromatic ring. The most preferred Z is substituted phenyl and substituted or unsubstituted thienyl.

The invention also includes optical isomers, diastereomers and enantiomers of the above structure. Thus, at all stereocenters where stereochemistry is not defined (C_{11}, C_{12}, C_{15}, and C_{16}), both epimers are envisioned. Preferred stereochemistry at all such stereocenters of the compounds of the invention mimic that of naturally occurring $PGF_{2\alpha}$.

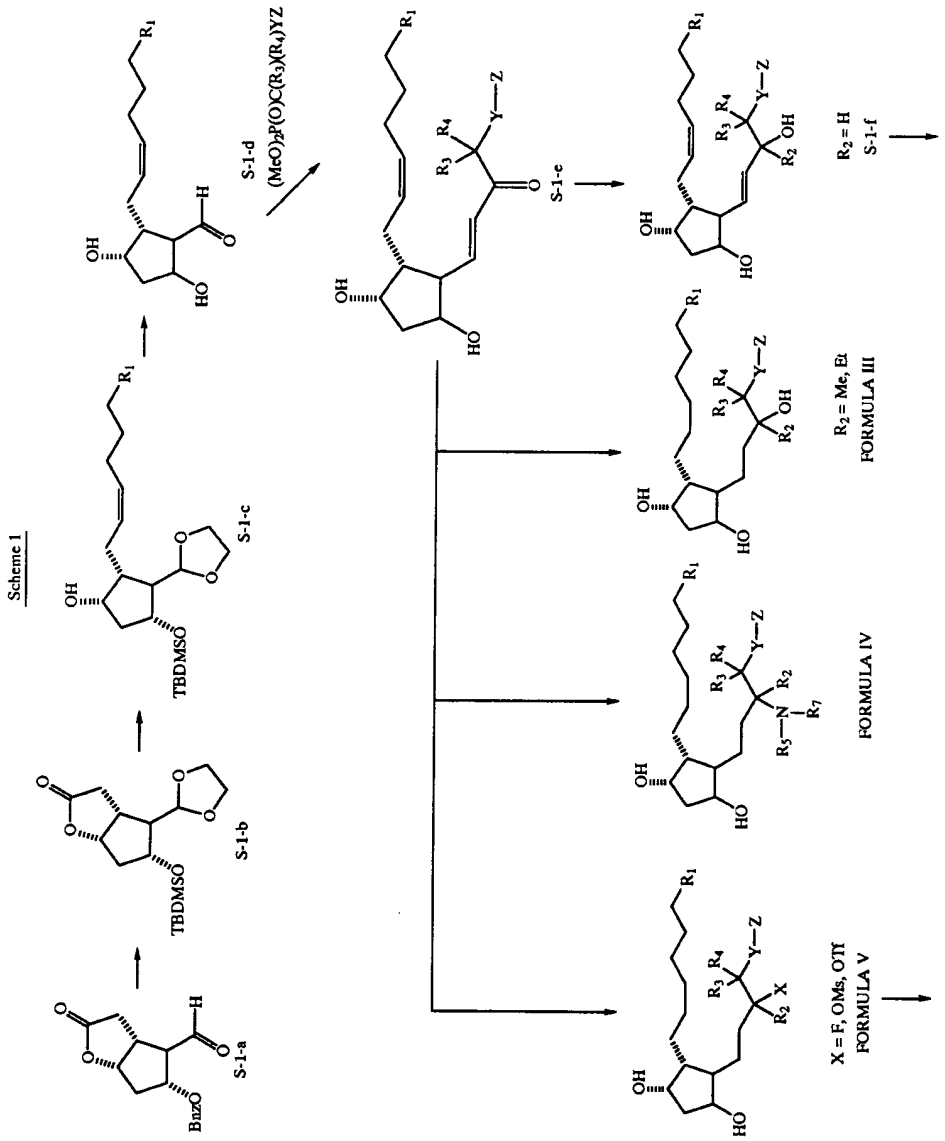
It has been discovered that the novel PGF analogs of the subject invention are useful for treating bone disorders, especially those that require a significant increase in bone mass, bone volume, or bone strength. Surprisingly, the compounds of the subject invention have been found to provide the following advantages over known bone disorder therapies: (1) An increase trabecular number through formation of new trabeculae; (2) An increase in bone mass and bone volume while maintaining a more normal bone turnover rate; and (3) An increase in bone formation at the endosteal surface without increasing cortical porosity.

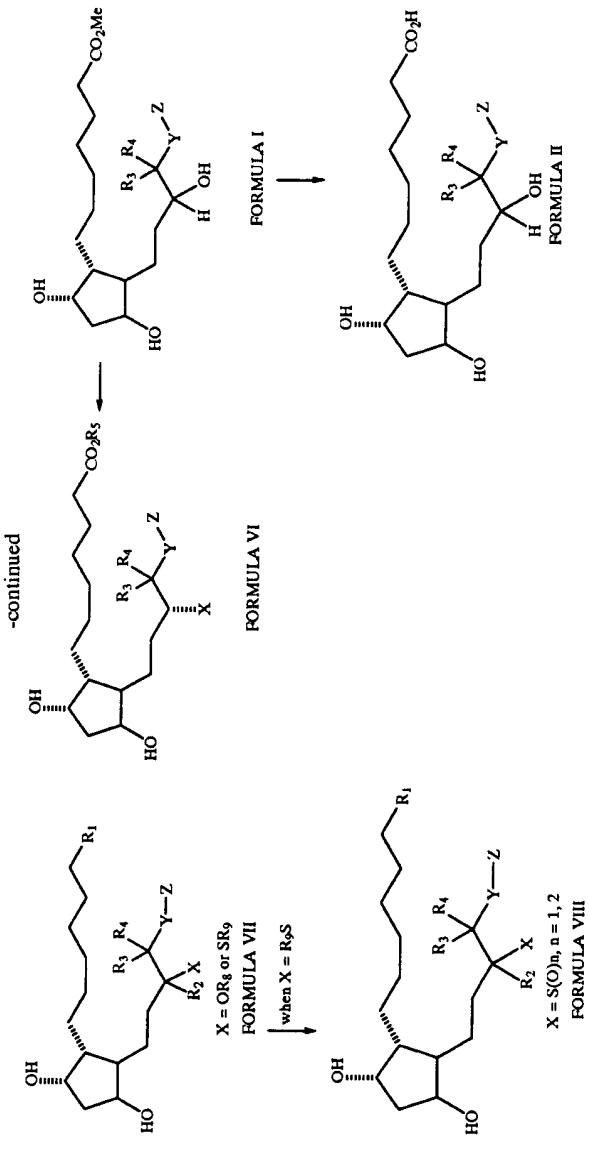
In order to determine and assess pharmacological activity, testing of the subject compounds in animals is carried out using various assays known to those skilled in the art. For example, the bone activity of the subject compounds can be conveniently demonstrated using an assay designed to test the ability of the subject compounds to increase bone volume, mass, or density. An example of such assays is the ovariectomized rat assay.

In the ovariectomized rat assay, six-month old rats are ovariectomized, aged 2 months, and then dosed once a day subcutaneously with a test compound. Upon completion of the study, bone mass and/or density can be measured by dual energy x-ray absorptometry (DXA) or peripheral quantitative computed tomography (pQCT), or micro computed tomography (mCT). Alternatively, static and dynamic histomorphometry can be used to measure the increase in bone volume or formation.

Pharmacological activity for glaucoma can be demonstrated using assays designed to test the ability of the subject compounds to decrease intraocular pressure. Examples of such assays are described in the following reference, incorporated herein: C. Iiljebris, G. Selen, B. Resul, J. Sternschantz, and U. Hacksell, "Derivatives of 17- Phenyl-18,19,20-trinorprostaglandin $F_{2\alpha}$ Isopropyl Ester: Potential Antiglaucoma Agents", *Journal of Medicinal Chemistry*, Vol. 38 No. 2 (1995), pp. 289–304.

Compounds useful in the subject invention can be made using conventional organic syntheses. A particularly preferred synthesis is the following general reaction scheme:


Scheme 1
S-1-a
S-1-b
S-1-c
S-1-d
S-1-e
S-1-f
TBDMSO
BnzO
(MeO)2P(O)C(R3)(R4)YZ
R2 = Me, Et
FORMULA III
R2 = H
FORMULA IV
X = F, OMs, OTf
FORMULA V


-continued
FORMULA I
FORMULA II
FORMULA VI
X = OR8 or SR9
FORMULA VII
when X = R9S
X = S(O)n, n = 1, 2
FORMULA VIII

In Scheme 1, R_1, R_2, R_3, R_4, X, Y, and Z are as defined above. The Corey Lactone (S1a) depicted as starting material for Scheme 1 is commercially available (such as from Sumitomo Chemical or Cayman Chemical).

Compounds depicted by S1f are available from compounds of the type depicted by S1e via standard reduction reactions. Compounds depicted by Formula I are available from compounds of S1f via simultaneous saturation of the double bonds of S1f. Compounds depicted by Formula I are exemplified in Examples 2, 4, 5, 7, 9, 11, 13, 16, 18, 20, 22, 24, 26, and 28. Compounds depicted by Formula II are prepared through a simple deesterification protocol of the compounds of Formula I. Compounds depicted by Formula II are exemplified in Examples 1, 3, 6, 8, 10, 12, 14, 15, 17, 19, 21, 23, 25, 27, and 29. Compounds depicted by Formula III can be prepared from compounds such of S1e via the addition of a carbon nucleophile followed by saturation and saponification. Compounds depicted by Formula III are exemplified in Examples 43 and 44. Compounds depicted by Formula IV can be prepared via imine formation followed by imine reduction, N-alkylation, hydrogenation, and saponification. Additional compounds depicted by Formula IV can be prepared via imine formation, as previously mentioned, followed by nucleophilic addition to the resulting imine followed by double bond saturation and saponification. Compounds depicted by Formula IV are exemplified in Examples 48, 49, and 50.

Compounds depicted by Formula V and Formula VII can be prepared through dihydroxyl protection of compounds of S1e followed by standard nucleophilic reduction of the ketone. The resulting free alcohol can be activated and displaced with nucleophiles such as, but not limited to, fluoride, alkoxide or sulfide to give compounds depicted by Formula V or Formula VII. Compounds depicted by Formula V are exemplified in Examples 36, 37, and 38. Compounds depicted by Formula VII are exemplified in Examples 39, 40, 41, 42, and 45. Compounds depicted by Formula VIII are prepared by the selective oxidation of compounds of Formula VII with the proviso that X must be sulfur. Compounds depicted by Formula VIII are exemplified in Examples 46 and 47. Compounds of the type depicted by Formula VI can be prepared from either compounds of Formula I or Formula II (compounds depicted by Formula II may require carboxylate activation) through nucleophilic addition to an activated carboxylate to produce an amide or new ester linkage to give the resulting hydroxamic acid, sulfonamide, or ester. Compounds depicted by Formula VI are exemplified in Examples 30–35.

The following non-limiting examples illustrate the compounds, compositions, and uses of the present invention.

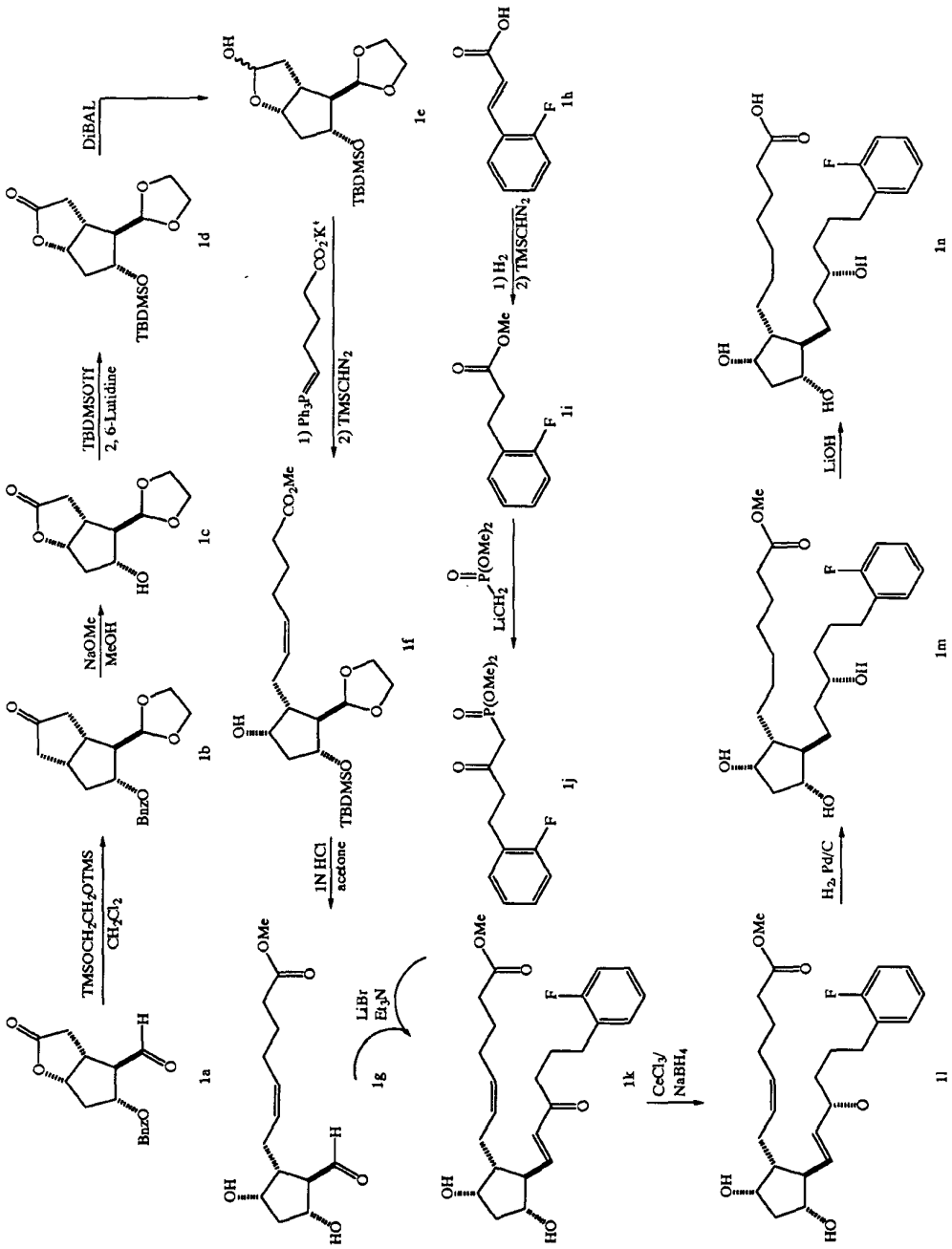
EXAMPLES

Compounds are analyzed using ^1H and ^{13}C NMR, Elemental analysis, mass spectra, high resolution mass spectra and/or IR spectra as appropriate.

Typically, inert solvents are used, preferably in dried form. For example, tetrahydrofuran (THF) is distilled from sodium and benzophenone, diisopropylamine is distilled from calcium hydride and all other solvents are purchased as the appropriate grade. Chromatography is performed on silica gel (70–230 mesh; Aldrich) or (230–400 mesh; Merck) as appropriate. Thin layer chromatography analysis is performed on glass mounted silica gel plates (200–300 mesh; Baker) and visualized using UV, 5% phosphomolybdic acid in EtOH, potassium permanganate in water, iodine, p-anisaldehyde in ethanol, or ammonium molybdate/cerric sulfate in 10% aqueous H_2SO_4.

Example 1

Preparation of 13,14-dihydro-17-(3-fluorophenyl)-17-trinor-prostaglandin $F_{1\alpha}$



a. 7-benzoyloxy-6-(2,5-dioxolanyl)-2-oxabicyclo[3.3.0] octan-3-one (1b):

In a round-bottomed flask equipped with a magnetic stir bar is placed 1,2-bis(trimethylsilyloxy)ethane (1.3 equiv.) in methylene chloride containing trimethysilyltrifluoromethanesulfonate (1 mL) at −78° C. To this is added, within 20 minutes, a solution of 1a (1 equiv) in CH_2Cl_2. The reaction is stirred for 1 hour at −78° C. and then slowly warmed to 25° C. for 1 hour. The reaction is quenched at 0° C. with water, extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated in vacuo to give crude 1b.

b. 6-(2,5-dioxolanyl)-7-hydroxy-2-oxabicyclo[3.3.0]octan-3-one (1c):

To a well stirred solution of crude 1b (1 equiv) in methanol at 0° C. is added a suspension of sodium methoxide (1.2 equiv) in MeOH. The reaction stirred at 0° C. for 1 hour and then warmed to 25° C. for 1 hour. The reaction is neutralized with acidic ion exchange resin which is washed thoroughly with MeOH. The filtrate is concentrated in vacuo to give a syrup which is subjected to flash chromatography on silica gel eluting with 4:1 hexane:ethyl acetate and 2% MeOH in CH_2Cl_2 to give 1c as a yellow syrup.

c. 6-(2,5dioxolanyl)-2-oxa-7-(1,1,2,2-tetramethyl-1-silapropoxy) bicyclo[3.3.0]octan-3-one (1d):

In a round-bottomed flask with a magnetic stir bar, is stirred a solution of 1c (1 equiv) in CH_2Cl_2. To this solution is added dropwise at −78° C. 2,6-lutidine (1.9 equiv) followed by TBDMSOTf (1.8 eq). The reaction stirred for 30 minutes at −78° C. and then warmed to 25° C. overnight. The reaction is quenched with water. The organic layer is washed with water, dried over $MgSO_4$, and concentrated in vacuo to give a yellow oil which is subjected to flash chromatography on silica gel eluting with hexanes then 1% MeOH in CH_2Cl_2. The product is then washed with 1N HCl, 0.1N HCl, water, and brine to give 1d.

d. 6-(2,5dioxolanyl)-2-oxa-7-(1,1,2,2-tetramethyl-1-silapropoxy) bicyclo[3.3.0]octan-2-ol (1e):

In a round-bottomed flask with a magnetic stir bar, is stirred a solution of 1d (1 equiv) in dry toluene. To this solution, at −78° C., is slowly added DIBAL (1.24 equiv). The reaction mixture is stirred for 2 hours and then warmed to 0° C. Saturated NH_4Cl is added to the reaction mixture which is then slowly warmed to 25° C. Diluted with water, the insoluble precipitate is removed by suction filtration and the solid is washed with EtOAc. The liquid phase is extracted with EtOAc and the combined organic phase is dried over $MgSO_4$ and concentrated in vacuo to give a yellow syrup. The product, 1e, must either be used immediately or stored at −70° C. overnight.

e. methyl 7-(5-(2,5-dioxolanyl)-2-hydroxy-4-(1,1,2,2-tetramethyl-(1-silapropoxy)cyclopentyl)hept-5-enoate (1f):

To a suspension of (4-carboxybutyl) triphenylphosphonium bromide (2.2 equiv) in THF at 0° C. under N_2 is added dropwise a solution of KHMDS (4.4 equiv). The resulting deep orange color reaction mixture is stirred for 1 hour at 25° C. To the reaction mixture above at −78° C. is added a solution of 1e (1 equiv) in THF. The reaction mixture is allowed to warm to 25° C. overnight. The reaction is quenched with water at 0° C. and the pH is adjusted to 3.5–4.0 with 1N HCl. The water phase is extracted with EtOAc and the combined organic phase is dried over $MgSO_4$ and is concentrated in vacuo to give a reddish-brown syrup containing crude acid. To a well stirred solution of crude acid in ether and MeOH at 0° C. is added TMS-diazomethane until a yellow color persists. The addition of 1 drop of glacial acetic acid, and thin layer chromatography verifies the reaction has gone to completion. The reaction solution is concentrated in vacuo and purified via flash chromatography on silica gel eluting with 30% EtOAc in hexanes yielding 1f.

f. methyl 7-(2,4-dihydroxy-5-formyl-cyclopentyl)hept-5-enoate (1g):

In a round-bottomed flask with a magnetic stir bar is placed an amount of the ketal, 1f. To this flask is added a sufficient amount of a mixture of 2 parts acetone to 1 part 1N HCl to bring the ketal completely into solution. This material is stirred until, by TLC, the starting material is consumed, typically overnight. The crude mixture, containing the product 1g, is extracted with ether, and the ether extract re-esterified in situ with, preferably, TMS-diazomethane. The organic extracts were concentrated under reduced pressure at 0° C. and used immediately without further purification.

g. Methyl 3-(2-fluorophenyl)propionate (1i):

In a Parr vessel is placed 2-fluorocinnamic acid (1h) (1.0 equiv) and palladium on carbon in a 1/1 methanol/ethyl acetate solution. The heterogeneous solution is placed on a Parr shaker and treated with hydrogen (50 psi) until uptake has ceased. The mixture is filtered through Celite and concentrated under reduced pressure. The residue is taken up in diethyl ether and treated with diazomethane until a yellow color persists. The solution is concentrated under reduced pressure to give the crude methyl ester. Purification is effected by column chromatography on silica gel (hexane/ethyl acetate 5/1) to yield Methyl 3-(2-fluorophenyl) propionate (1i) in quantitative yield.

h. Dimethyl-4-(2-fluorophenyl)-2-oxo-butylphosphonate (1j):

In a flame-diried, round-bottomed flask equipped with a stir bar and thermometer is placed dimethylmethyl phosphonate (1.0 equiv.) in anhydrous THF. The solution is cooled to −78° C. and treated with n-butyllithium (1.05 equiv.). The reaction mixture is allowed to stir for 15 minutes. To this solution is added methyl-3-(2-fluorophenyl) propionate (1.1 equiv.) in anhydrous THF. The mixture is allowed to warm to room temperature over the next 6 hours. The mixture is treated with a saturated solution of ammonium chloride and extracted with CH_2Cl_2. The organic layer is washed with water followed by brine. The combined aqueous layers are back extracted with CH_2Cl_2 and the organic layers combined, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Purification is effected by silica gel column chromatography (hexane/ethyl acetate/2-propanol 45/50/5 to hexane/ethyl acetate/2-propanol 40/50/10) to yield 1.34 g (70%) of dimethyl-4-(2-fluorophenyl)-2-oxo-butylphosphonate (1j) as an oil.

i. 17-(2-fluorophenyl)-17-trinor-15-oxo-prostaglandin $F_{2\alpha}$ methyl ester (1k):

In a flame-dried, round-bottomed flask equipped with a magnetic stirbar is placed dimethyl-4-(2-fluorophenyl)-2-oxo-butylphosphonate (1j) (1.43 equiv) in DME and water. To this solution is added lithium bromide (1.65 equiv), triethylamine (1.65 equiv), and methyl 7-(2-formyl-3,5-dihydroxycyclopentyl)hept-5-enoate (1g) (1.0 equiv). The solution is stirred at room temperature for 48 hours. At this point additional triethylamine and water is added and the solution is stirred for an additional hour. The solution is poured into brine and extracted with 3 portions of ethyl acetate. The organic layers are combined, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Purification is effected by silica gel column chromatography (dichloromethane/methanol 19/1) to give 17-

(2-fluorophenyl)-17-trinor-15-oxo-prostaglandin $F_{2\alpha}$ methyl ester (1k) as an oil.

j. 15-(R,S)-17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{2\alpha}$ methyl ester (1l):

In a flame-dried round-bottomed flask equipped with a stir bar is placed 17-(2-fluorophenyl)-17-trinor-15-oxo-prostaglandin $F_{2\alpha}$ methyl ester (1k) (1.0 equiv), cerium trichloride (1.05 equiv) in methanol. The solution is stirred at room temperature for 5 minutes. The solution is cooled to −10° C. and sodium borohydride (1.02 equiv.) in methanol is added. The solution is stirred at −10° C. for 3 hours. The mixture is treated with water and the pH brought to 6–7 with 1N hydrochloric acid. The mixture is extracted twice with ethyl acetate, and the organic layers combined, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. Purification was effected by silica gel column chromatography (3% methanol in dichloromethane to 5% methanol in dichloromethane) to give (43%) of the 15 (R) epimer and (19.6%) of the 15 (S) epimer as colorless oils.

k. 13,14-dihydro-17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{1\alpha}$ methyl ester (1m):

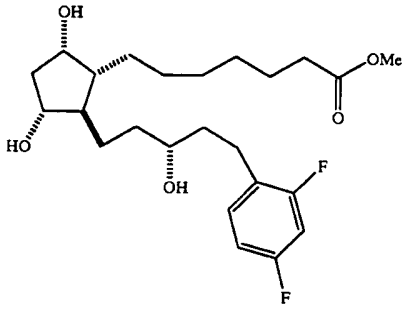
In a flame-dried round-bottomed flask equipped with a stir bar was placed 17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{2\alpha}$ methyl ester (1l) (1.0 equiv.) and palladium on carbon in ethyl acetate (3 mL). The heterogeneous mixture is treated with hydrogen via a balloon for 18 hours. The mixture is filtered through Celite and concentrated under reduced pressure to give a quantitative yield 13,14-dihydro-17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{1\alpha}$ methyl ester (1m).

l. 13,14-dihydro-17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{1\alpha}$ methyl ester (1n):

In a round-bottomed flask equipped with a stir bar is placed 13,14-dihydro-17-(2-fluorophenyl)-17-trinor-prostaglandin $F_{1\alpha}$ methyl ester (1m) (1.0 equiv) and lithium hydroxide monohydrate (1.8 equiv) in a 50/50 THF water solution. The mixture is stirred at room temperature for 6 hours and then diluted with water and acidified to pH 2–3 with 1N HCl. The aqueous phase is extracted 3 times with ethyl acetate and the organic layers combined. The combined organics were dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to yield the crude acid. Purification was effected by HPLC to yield (41%) of an analytical sample. Utilizing substantially the method of Example 1 (and using the appropriate starting materials), the following subject compounds of Examples 2–29 are obtained.

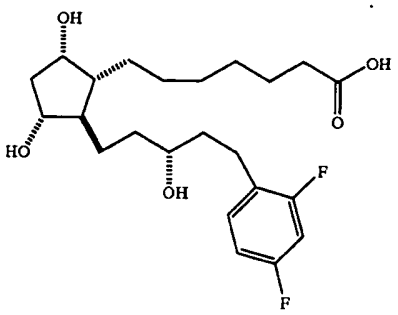
Example 2

13,14-dihydro-17-(2,4difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



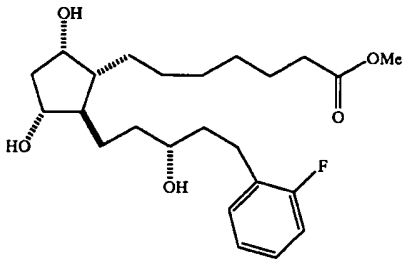
Example 3

13,14-dihydro-17-(2,4difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$



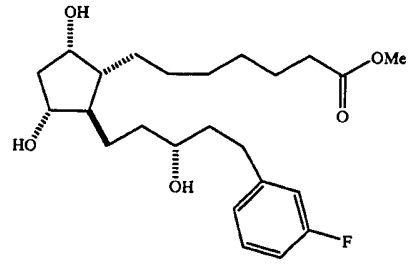
Example 4

13,14-dihydro-17-(2-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



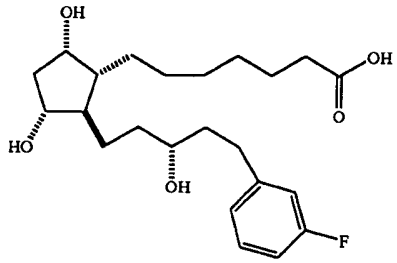
Example 5

13,14-dihydro-17-(3-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



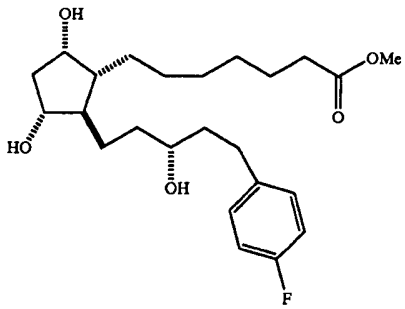
Example 6

13,14-dihydro-17-(3-fluorophenyl-17-trinor prostaglandin $F_{1\alpha}$



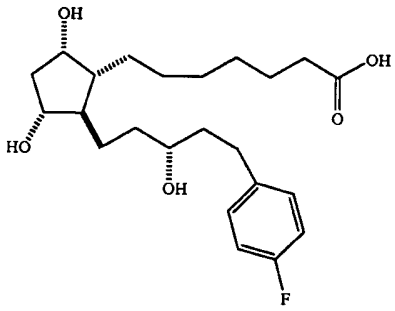
Example 7

13,14-dihydro-17-(4-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



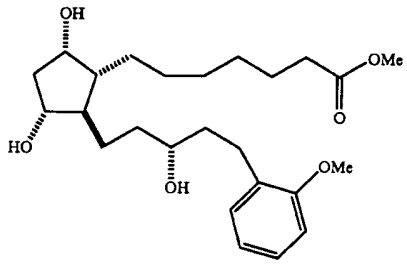
Example 8

13,14-dihydro-17-(4-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$



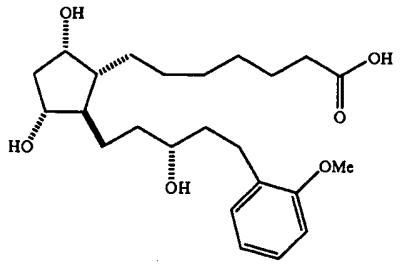
Example 9

13,14-dihydro-17-(2-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



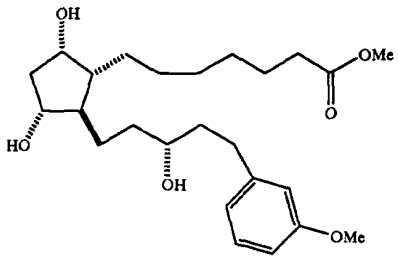
Example 10

13,14-dihydro-17-(2-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



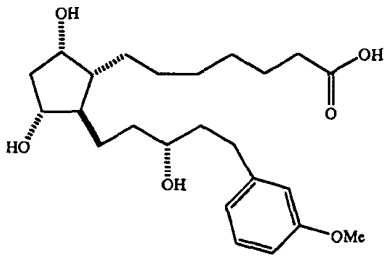
Example 11

13,14-dihydro-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



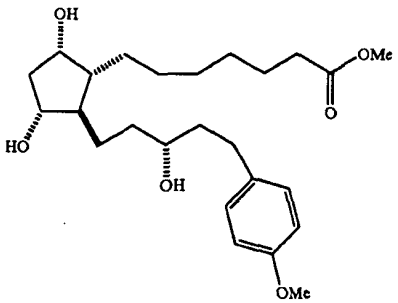
Example 12

13,14-dihydro-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



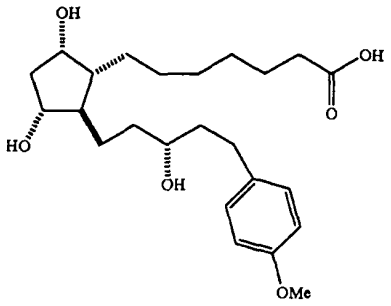
Example 13

13,14-dihydro-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



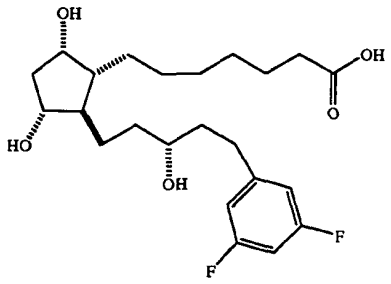
Example 14

13,14-dihydro-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



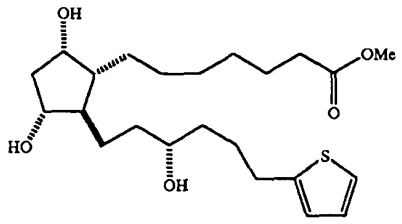
Example 15

13,14-dihydro-17-(3,5-difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$



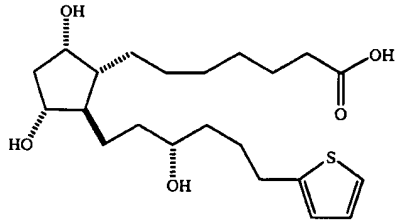
Example 16

13,14-dihydro-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$ methyl ester



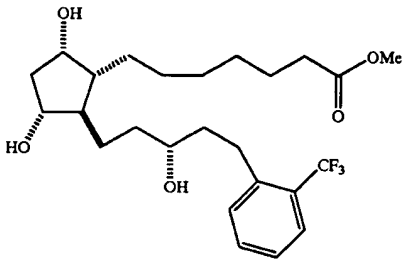
Example 17

13,14-dihydro-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$



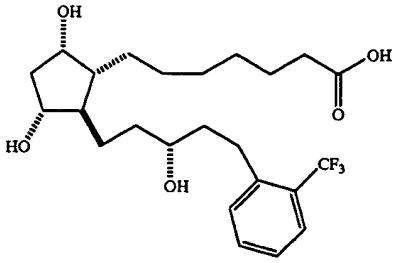
Example 18

13,14-dihydro-17-((2-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



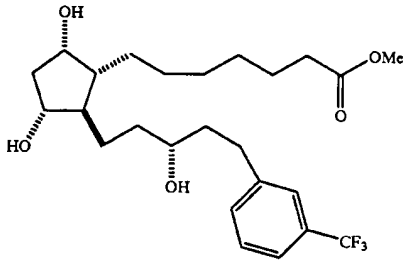
Example 19

13,14-dihydro-17-((2-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$



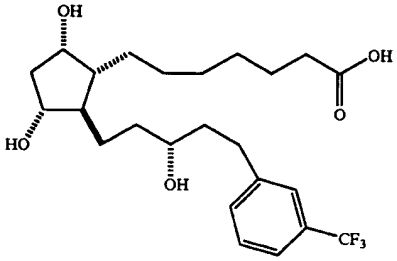
Example 20

13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



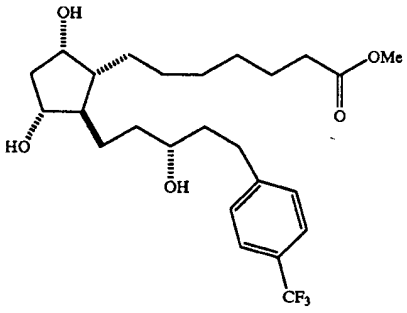
Example 21

13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17trinor prostaglandin $F_{1\alpha}$



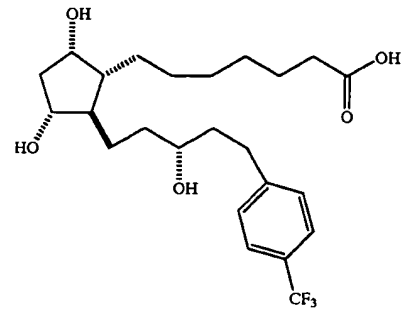
Example 22

13,14-dihydro-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$methyl ester



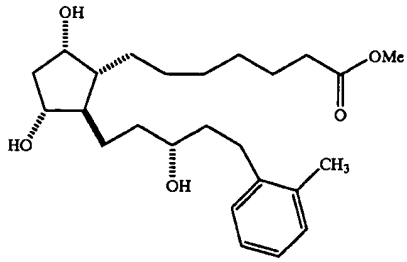
Example 23

13,14-dihydro-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$



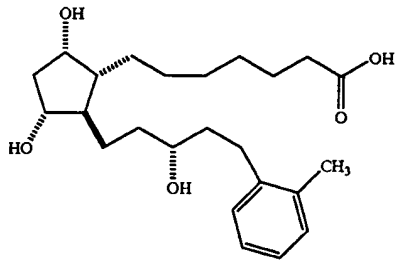
Example 24

13,14-dihydro-17-(2-methylphenyl-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



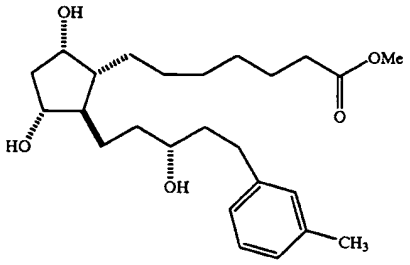
Example 25

13,14-dihydro-17-(2-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$



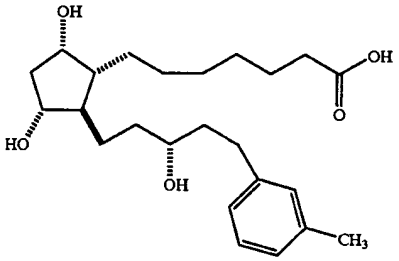
Example 26

13,14-dihydro-17-(3-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



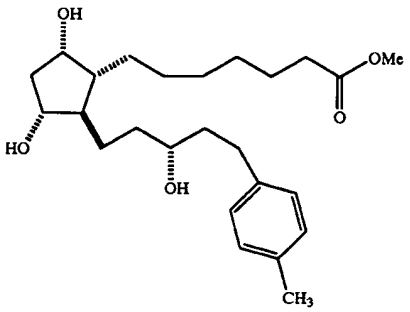
Example 27

13,14-dihydro-17-(3-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$



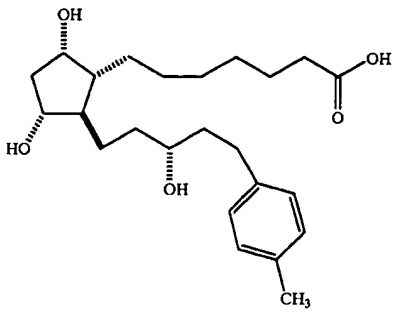
Example 28

13,14-dihydro-17-(4-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester



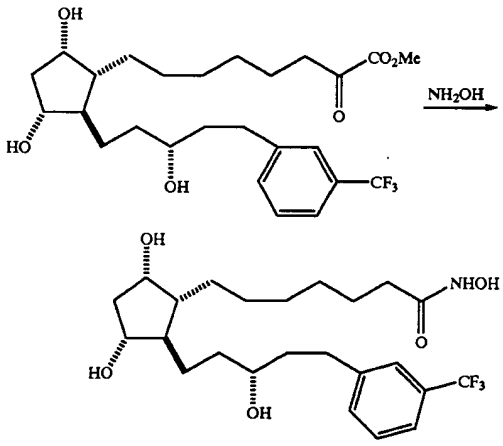
Example 29

13,14-dihydro-17-(4-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$



Example 30

13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-hydroxamic acid



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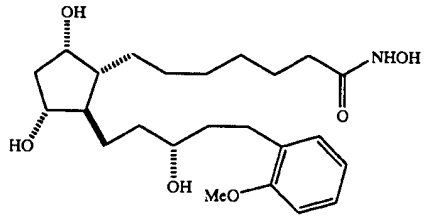
To a solution of 13,14-dihydro-17-(3-trifluoromethyl)-phenyl trinor prostaglandin $F_{1\alpha}$ methyl ester (Example 20) in methanol is added hydroxylamine in basic methanol (1.25 equiv.). The solution is stirred at room temperature for 18 hours. The solution is treated with 1N hydrochloric acid and extracted with ethyl acetate. The organic layer is washed with brine and dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The residue is purified by HPLC to yield 13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-hydroxamic acid.

Utilizing substantially the method of Example 30 (and using the appropriate ester), the following subject compounds of Examples 31 and 32 are obtained.

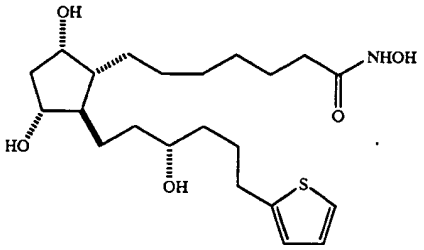
Example 31

13,14-dihydro-17-(2-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-hydroxamic acid



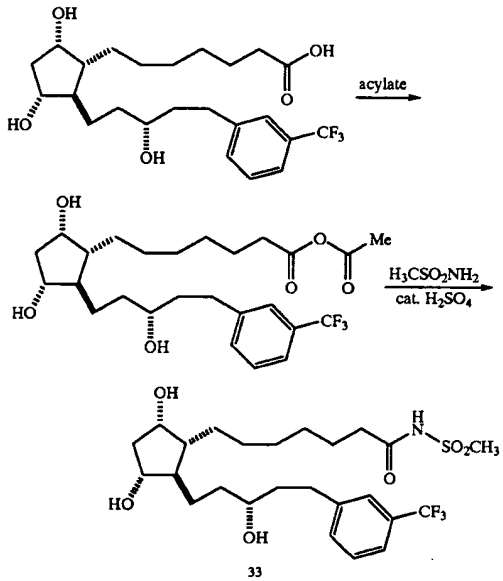
Example 32

13,14-dihydro-18-(2-thienyl)-dinor prostaglandin $F_{1\alpha}$-1-hydroxamic acid



Example 33

13,14-dihydro-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-sulfonamide



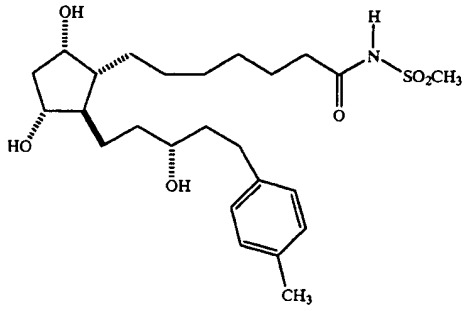
33

Example 23 is converted to the anhydride followed by treatment with methanesulfonylamide as disclosed in A. D. Kemp and H. Stephen, *J. Chem. Soc.* (1948) p. 110.

Utilizing substantially the method of Example 33 (and using the appropriate acid), the following subject compounds of Examples 34 and 35 are obtained.

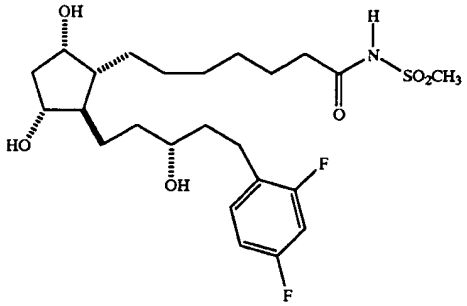
Example 34

13,14-dihydro-17-(4-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-sulfonamide



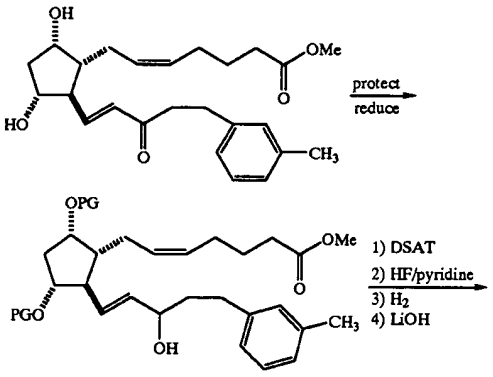
Example 35

13,14-dihydro-17-(2,4difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$-1-sulfonamide



Example 36

13,14-dihydro-15-fluoro-17-(3-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$



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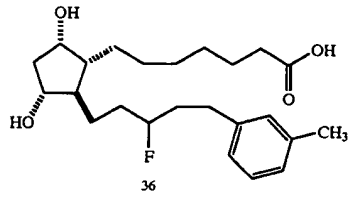
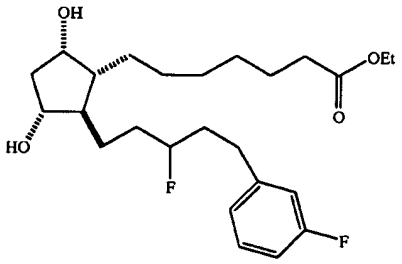


The precursor to Example 27 corresponding to 1k from Example 1 is protected and reduced to give the 9,11-protected bis ether. The resulting compound is treated with diethylaminosulfur trifluoride (DSAT) (as disclosed in the following references: *Org. React.* Vol. 35 (1988) p. 513; *J. Org. Chem.* Vol. 40 (1975) p. 574; and references cited therein) to give 13,14-dihydro-15-fluoro-17-(3-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$ after the appropriate transformation as described in Example 1.

Examples 37 and 38 are prepared in a manner substantially similar to Example 36 using the appropriate intermediate corresponding to 1k (from Example 5 and Example 25 respectively) in Example 1 followed by standard esterification with the appropriate alcohol.

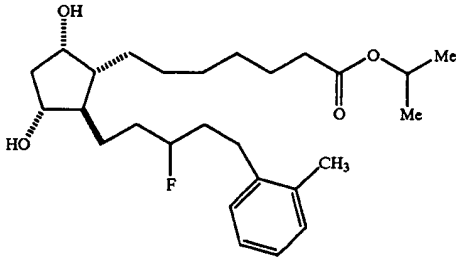
Example 37

13,14-dihydro-15-fluoro-17-(3-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ ethyl ester



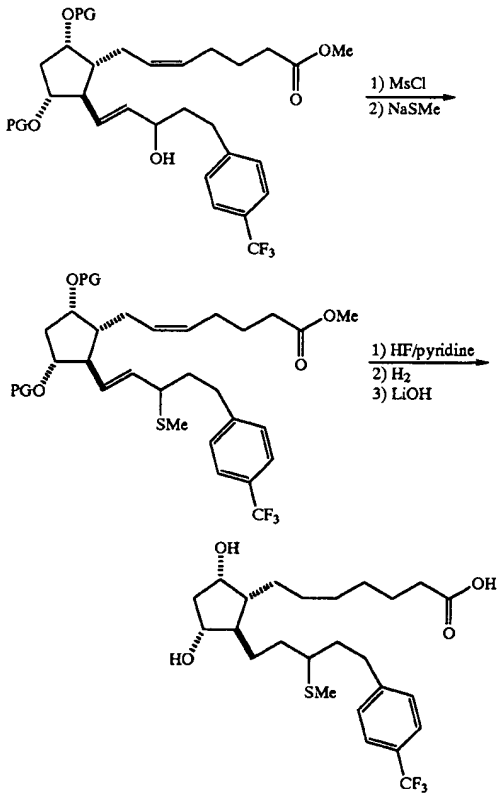
Example 38

13,14-dihydro-15-fluoro-17-(2-methylphenyl)-17-trinor prostaglandin $F_{1\alpha}$ isopropyl ester



Example 39

13,14-dihydro-15-methylthio-17-((4-trifluoromethyl) phenyl)-17-trinor prostaglandin $F_{1\alpha}$



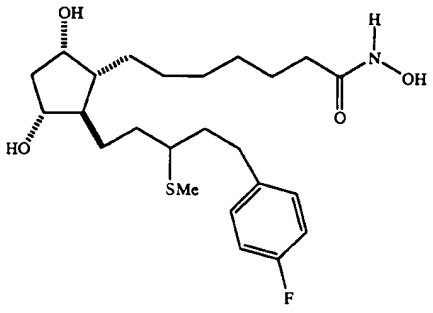
39

The precursor to Example 23 corresponding to 1k from Example 1 is protected and reduced to give the 9,11-protected bis ether. This compound is treated with methanesulfonyl chloride (1.2 equiv) and base (1.2 equiv) (as disclosed in the following references: *J.C.S. Chem. Comm.* (1975) p. 658; *Tetrahedron Lett.* (1975) p. 3183; and references cited therein) to generate the intermediate mesylate, which is then treated immediately with nucleophiles (sodium thiomethoxide) (as disclosed in *Tetrahedron Lett.* Vol. 23 (1982) p. 3463 and references cited therein.) to give the protected thioalkyl ether. Subsequent transformation as described in Example 1 provides 13,14-dihydro-15-methylthio-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$.

Example 40 is prepared in a manner substantially similar to Example 39 (from a precursor corresponding to 1k from Example 7) followed by conversion to the hydroxamic acid as shown in Example 30.

Example 40

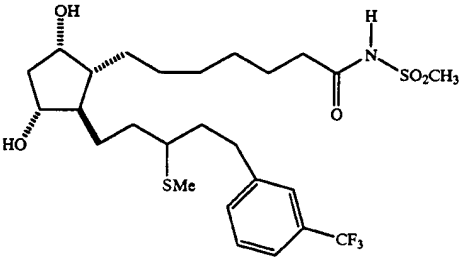
13,14-dihydro-15-methylthio-17-(4-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ hydroxamic acid



Example 41 is prepared in a substantially similar manner as Example 39 (from a precursor corresponding to 1k from Example 21) followed by conversion to the sulfonamide as shown in Example 33.

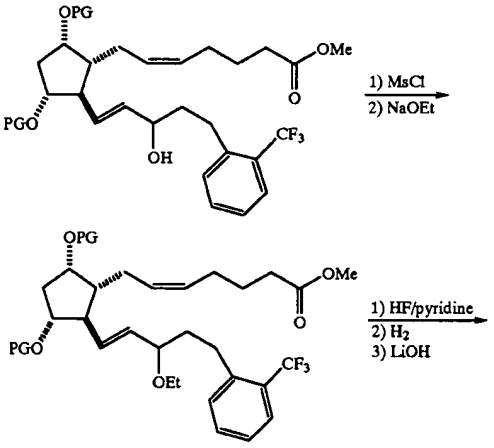
Example 41

13,14-dihydro-15-methylthio-17-((3-trifluoromethyl) phenyl)-17-trinor prostaglandin $F_{1\alpha}$-sulfonamide



Example 42

13,14-dihydro-15-ethoxy-17-((2-trifluoromethyl) phenyl)-17-trinor prostaglandin $F_{1\alpha}$



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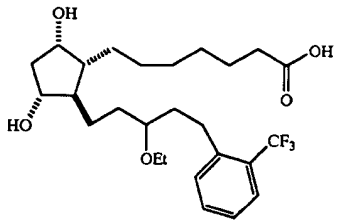
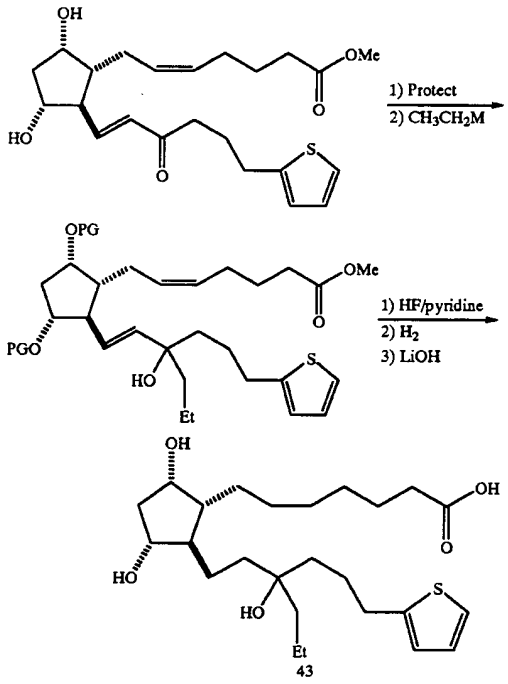


42

The precursor to Example 19 corresponding to 1k from Example 1 is protected and reduced to give the 9,11-protected bis ether. This compound is treated with methanesulfonyl chloride (1.2 equiv.) and base (1.2 equiv.) (as disclosed in the following references: *J.C.S. Chem. Comm.* (1975) p. 658; *Tetrahedron Lett.* (1975) p. 3183; and references cited therein.) to generate the intermediate mesylate, which is then treated immediately with sodium ethoxide to give the protected alkyl ether. Subsequent transformation as described in Example 1 provides 13,14-dihydro-15-ethoxy-17-((2-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$.

Example 43

13,14-dihydro-15-ethyl-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$



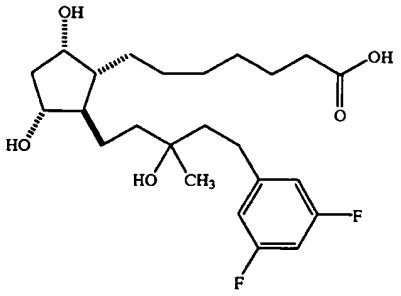
43

The precursor to Example 17 corresponding to 1k from Example 1 is protected and reduced to give the 9,11-protected bis ether. The resulting protected diol is treated with one of a variety of carbon nucleophiles, such as ethyl magnesium bromide to give the resulting tertiary alcohol. Deprotection followed by the transformation outlined in Example 1 provides 13,14-dihydro-15-ethyl-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$.

Utilizing substantially the method of Example 43 (and using the appropriate carbon nucleophile), the following subject compound of Example 44 is obtained.

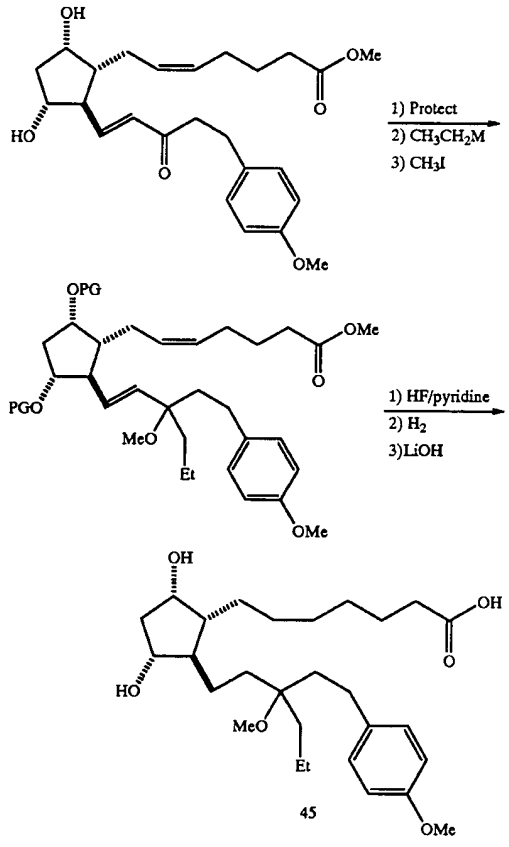
Example 44

13,14-dihydro-15-methyl-17-(3,5-difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$



Example 45

13,14-dihydro-15-ethyl-15-methoxy-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



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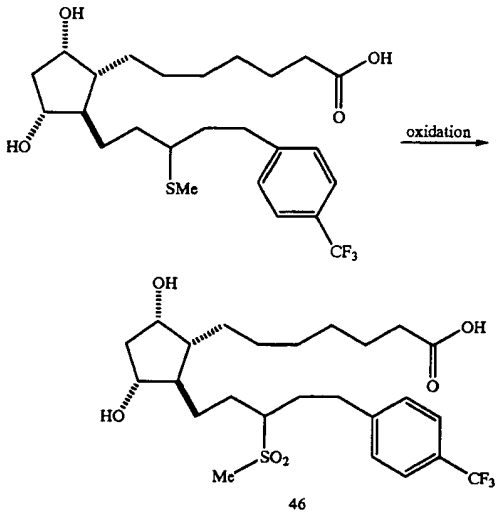
The compound of Example 45 is prepared by utilizing the protocol outlined in Example 43 (from the precursor corresponding to 1k for Example 13) followed by O-alkylation of

the resulting C_{15} alkoxide with a variety of alkyl halides (iodomethane in this example). This is followed by deprotection, hydrogenation, and saponificiation as outlined in Example 43 and Example 1 to give 13,14-dihydro-15-ethyl-15-methoxy-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$

Example 46

13,14-dihydro-15-sulfonylmethyl-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$



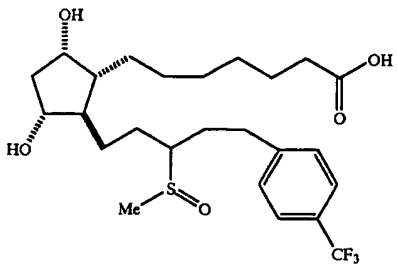
46

The thiomethyl ether of Example 39 is treated with the appropriate oxidizing agent as disclosed in the following references: *Tetrahedron Lett.* (1982) p. 3467; *Prostaglandins* Vol. 24 (1982) p. 801; *Tetrahedron Lett.* Vol. 23 (1982) p. 1023; and references cited therein.

Utilizing substantially the method of Example 46 (and using the appropriate thioether), the following subject compound of Example 47 is obtained.

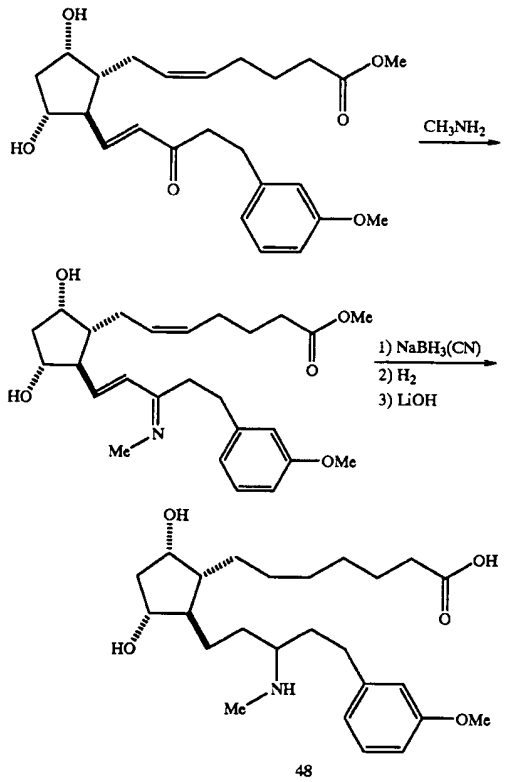
Example 47

13,14-dihydro-15-sulfoxylmethyl-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$



Example 48

13,14-dihydro-15-N-methylamino-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$

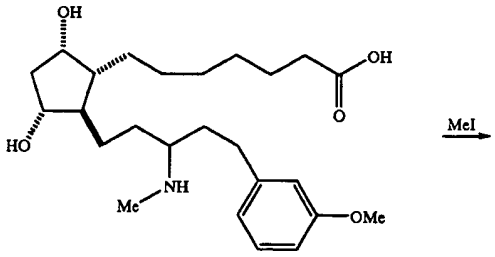


48

The intermediate of Example 12 corresponding to 1k is condensed with methyl amine followed by reduction with sodium cyanoborohydride to give 13,14-dihydro-15-N-methylamino-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$, after saponification and deprotection.

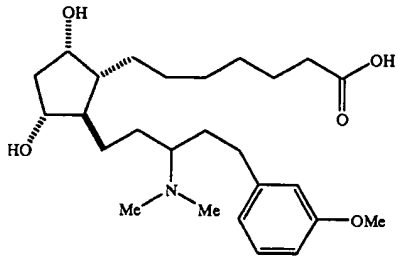
Example 49

13,14-dihydro-15-N,N'-dimethylamino-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



48

-continued

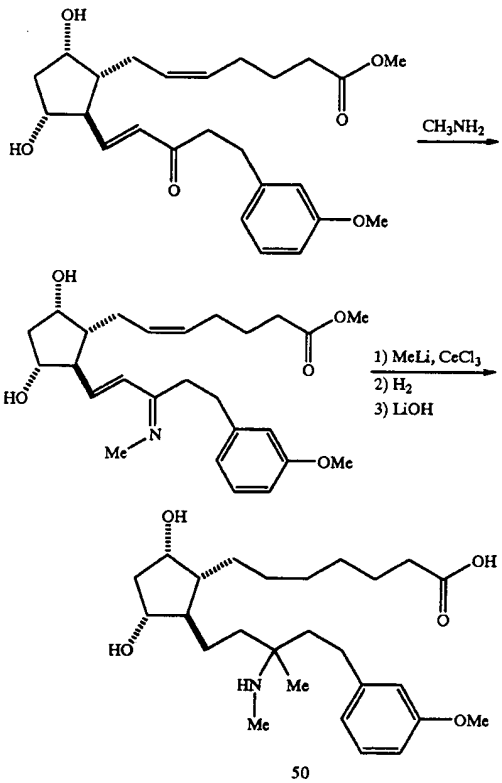


49

The compound of Example 49 is prepared from the compound of Example 48 by simple alkylation with iodomethane.

Example 50

13,14-dihydro-15-aminomethyl-15-methyl-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$



50

The intermediate imine of Example 48 is treated with methylcerium (excess) (for examples of cerium-mediated nucleophilic additions see the following references: *J. Org. Chem.*, Vol. 49 (1984) p. 3904; *J. Am. Chem. Soc.*, Vol. 111 (1989) p. 4392; and references therein) to give 13,14-dihydro-15-aminomethyl-15-methyl-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ after hydrogenation and saponification as described in Example 1.

Compositions

Compositions of the subject invention comprise a safe and effective amount of the subject compounds, and a pharmaceutically-acceptable carrier. As used herein, "safe and effective amount" means an amount of a compound sufficient to significantly induce a positive modification in the condition to be treated, but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical judgment. A safe and effective amount of a compound will vary with the particular condition being treated, the age and physical condition of the patient being treated, the severity of the condition, the duration of the treatment, the nature of concurrent therapy, the particular pharmaceutically-acceptable carrier utilized, and like factors within the knowledge and expertise of the attending physician.

In addition to the compound, the compositions of the subject invention contain a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier", as used herein, means one or more compatible solid or liquid filler diluents or encapsulating substances which are suitable for administration to a subject. The term "compatible", as used herein, means that the components of the composition are capable of being commingled with the compound, and with each other, in a manner such that there is no interaction which would substantially reduce the pharmaceutical efficacy of the composition under ordinary use situations. Pharmaceutically-acceptable carriers must, of course, be of sufficiently high purity and sufficiently low toxicity to render them suitable for administration to the subject being treated.

Some examples of substances which can serve as pharmaceutically-acceptable carriers or components thereof are sugars, such as lactose, glucose and sucrose; starches, such as cornstarch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, cellulose acetate; powdered tragacanth; malt; gelatin; talc; solid lubricants, such as stearic acid, magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerin, sorbitol, mannitol, and polyethylene glycol; alginic acid; emulsifiers, such as the Tweens®; wetting agents such as sodium lauryl sulfate; coloring agents; flavoring agents, excipients; tableting agents; stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline; and phosphate buffer solutions.

The choice of a pharmaceutically-acceptable carrier to be used in conjunction with a compound is basically determined by the way the compound is to be administered. The compounds of the present invention may be administered systemically. Routes of administration include transdermal; oral; parenterally, including subcutaneous or intravenous injection; topical; and/or intranasal.

The appropriate amount of the compound to be used may be determined by routine experimentation with animal models. Such models include, but are not limited to the intact and ovariectomized rat models, the ferret, canine, and non human primate models as well as disuse models.

Preferred unit dosage forms for injection include sterile solutions of water, physiological saline, or mixtures thereof. The pH of said solutions should be adjusted to about 7.4. Suitable carriers for injection or surgical implants include hydrogels, controlled- or sustained release devises, polylactic acid, and collagen matrices.

Suitable pharmaceutically-acceptable carriers for topical application include those suited for use in lotions, creams, gels and the like. If the compound is to be administered perorally, the preferred unit dosage form is tablets, capsules and the like. The pharmaceutically-acceptable carriers suitable for the preparation of unit dosage forms for oral

administration are well-known in the art. Their selection will depend on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of the subject invention, and can be made without difficulty by those skilled in the art.

Methods of Use

The compounds of the present invention are useful in treating many medical disorders, including for example, ocular disorders, hypertension, fertility control, nasal congestion, neurogenic bladder disorder, gastrointestinal disorders, dermatological disorders, and osteoporosis.

The compounds of the present invention are useful in increasing bone volume and trabecular number through formation of new trabeculae, increasing bone mass while maintaining a normalized bone turnover rate, and formation of bone at the endosteal surface without removing bone from the existing cortex. Thus, these compounds are useful in the treatment and prevention of bone disorders.

The preferred routes of administration for treating bone disorders are transdermal and intranasal. Other preferred routes of administration include rectal, sublingual, and oral.

The dosage range of the compound for systemic administration is from about 0.01 to about 1000 μg/kg body weight, preferably from about 0.1 to about 100 μg/kg per body weight, most preferably from about 1 to about 50 μg/kg body weight per day. The transdermal dosages will be designed to attain similar serum or plasma levels, based upon techniques known to those skilled in the art of pharmacokinetics and transdermal formulations. Plasma levels for systemic administration are expected to be in the range of 0.01 to 100 nanograms/ml, more preferably from 0.05 to 50 ng/ml, and most preferably from 0.1 to 10 ng/ml. While these dosages are based upon a daily administration rate, weekly or monthly accumulated dosages may also be used to calculate the clinical requirements.

Dosages may be varied based on the patient being treated, the condition being treated, the severity of the condition being treated, the route of administration, etc. to achieve the desired effect.

The compounds of the present invention are also useful in decreasing intraocular pressure. Thus, these compounds are useful in the treatment of glaucoma. The preferred route of administration for treating glaucoma is topically.

Composition and Method Examples

The following non-limiting examples illustrate the subject invention. The following composition and method examples do not limit the invention, but provide guidance to the skilled artisan to prepare and use the compounds, compositions and methods of the invention. In each case other compounds within the invention may be substituted for the example compound shown below with similar results. The skilled practitioner will appreciate that the examples provide guidance and may be varied based on the condition being treated and the patient.

Example A

Pharmaceutical compositions in the form of tablets are prepared by conventional methods, such as mixing and direct compaction, formulated as follows:

Ingredient	Quantity (mg per tablet)
Compound of Example 1	5
Microcrystalline Cellulose	100
Sodium Starch Glycollate	30
Magnesium Stearate	3

When administered orally once daily, the above composition substantially increases bone volume in a patient suffering from osteoporosis.

Example B

Pharmaceutical compositions in liquid form are prepared by conventional methods, formulated as follows:

Ingredient	Quantity
Compound of Example 1	5 mg
Phosphate buffered physiological saline	10 ml
Methyl Paraben	0.05 ml

When 1.0 ml of the above composition is administered subcutaneously once daily, the above composition substantially increases bone volume in a patient suffering from osteoporosis.

Example C

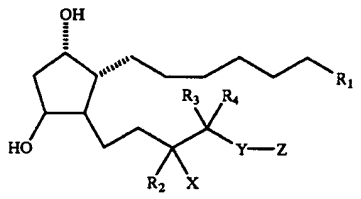
Topical pharmaceutical compositions for lowering intraocular pressure are prepared by conventional methods and formulated as follows:

Ingredient	Amount (wt %)
Compound of Example 38	0.004
Dextran 70	0.1
Hydroxypropyl methylcellulose	0.3
Sodium Chloride	0.77
Potassium chloride	0.12
Disodium EDTA (Edetate disodium)	0.05
Benzalkonium chloride	0.01
HCL and/or NaOH	pH 7.2–7.5
Purified water	q.s. to 100%

While particular embodiments of the subject invention have been described, it would be obvious to those skilled in the art that various changes and modifications to the compositions disclosed herein can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of this invention.

What is claimed is:

1. A compound having the structure:



wherein

(a) R_1 is CO_2H, $C(O)NHOH$, CO_2R_5, CH_2OH, $S(O)_2R_5$, $C(O)NHR_5$, $C(O)NHS(O)_2R_5$, or tetrazole; wherein R_5

is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(b) R_2 is H or lower alkyl;

(c) X is NR_6R_7, OR_8, SR_9, $S(O)R_9$, $S(O)_2R_9$, or F; wherein R_6, R_7, and R_8 are independently selected from the group consisting of H, acyl, alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, and heteroaromatic ring; and wherein R_9 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(d) R_3 and R_4 are independently H, CH_3, C_2H_5, OR_{10}, SR_{10}, or OH, except that both R_3 and R_4 are not OH; wherein R_{10} is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring, R_{10} having from 1 to about 8 member atoms;

(e) Y is $(CH_2)_n$; n being an integer from 1 to about 3;

(f) Z is heterocyclic aliphatic ring, monocyclic heteroaromatic ring, or substituted phenyl when n is 2 or 3 wherein said phenyl substituents are selected from the group consisting of halo, cyano, heteroalkyl, haloalkyl, and phenyl; and Z is heterocyclic aliphatic ring or substituted phenyl when n is 1 wherein said phenyl substituents are selected from the group consisting of halo, cyano, heteroalkyl, haloalkyl, and phenyl; wherein heteroalkyl is a saturated or unsaturated chain containing carbon and at least one heteroatom; and

any optical isomer, diasteroemer, enantiomer of the above structure, or a pharmaceutically-acceptable salt, or biohydrolyzable amide, ester, or imide thereof.

2. The compound according to claim **1** wherein R_1 is selected from the group consisting of CO_2H, C(O)NHOH, CO_2CH_3, $CO_2C_2H_5$, $CO_2C_3H_7$, $CO_2C_4H_9$, $CO_2C_3H_7O_2$, and $C(O)NHS(O)_2R_5$.

3. The compound according to claim **2** wherein R_2 is H or CH_3.

4. The compound according to claim **3** wherein X is OR_8 or NR_6R_7.

5. The compound according to claim **4** wherein Z is monocyclic.

6. The compound according to claim **5** wherein R_1 is selected from the group consisting of CO_2H, C(O)NHOH, CO_2CH_3, and $CO_2C_3H_5$.

7. The compound according to claim **6** wherein X is OH.

8. The compound according to claim **7** wherein n is 2 or 3 and Z is substituted phenyl or heteroaromatic ring.

9. The compound according to claim **8** wherein Z is substituted phenyl or substituted or unsubstituted thienyl.

10. The compound according to claim **9** wherein n is 2.

11. The compound according to claim **9** wherein Z is substituted with from 1 to about 4 substituents, said substituents being selected independently from the group consisting of halo, alkyl, haloalkyl, cyano, nitro, alkoxy, phenyl, and phenoxy.

12. The compound according to claim **1** wherein Z is substituted with from 1 to about 4 substituents, said substituents being selected independently from the group consisting of halo, alkyl, haloalkyl, cyano, nitro, alkoxy, phenyl, and phenoxy.

13. The compound according to claim **1** wherein said compound is selected from the group consisting of:

13,14-dihydro-17-(2,4-difluorophenyl)-17- trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(2,4-difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(2-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(2-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(3-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(3-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(4-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(4-fluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(2-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(2-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(3-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-(4-methoxyphenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-(3,5-difluorophenyl)-17-trinor prostaglandin $F_{1\alpha}$.

14. The compound according to claim **1** wherein said compound is selected from the group consisting of:

13,14-dihydro-17-((2-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-((2-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

13,14-dihydro-17-((3-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$;

13,14-dihydro-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$ methyl ester;

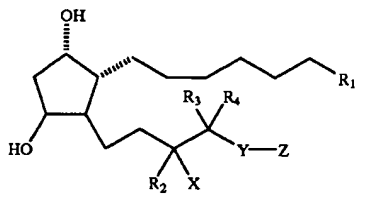
13,14-dihydro-17-((4-trifluoromethyl)phenyl)-17-trinor prostaglandin $F_{1\alpha}$.

15. The compound according to claim **1** wherein said compound is selected from the group consisting of:

13,14-dihydro-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$, methyl ester;

13,14-dihydro-18-(2-thienyl)-18-dinor prostaglandin $F_{1\alpha}$.

16. A method of treating a human or other animal subject having a bone disorder, said method comprising administering to said subject a compound according to the structure:



wherein

(a) R_1 is CO_2H, C(O)NHOH, CO_2R_5, CH_2OH, $S(O)_2R_5$, $C(O)NHR_5$, $C(O)NHS(O)_2R_5$, or tetrazole; wherein R_5 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(b) R_2 is H or lower alkyl;

(c) X is NR_6R_7, OR_8, SR_9, $S(O)R_9$, $S(O)_2R_9$, or F; wherein R_6, R_7, and R_8 are independently selected from the group consisting of H, acyl, alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, and heteroaromatic ring; and wherein R_9 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(d) R_3 and R_4 are independently H, CH_3, C_2H_5, OR_{10}, SR_{10}, or OH, except that both R_3 and R_4 are not OH; wherein R_{10} is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring, R_{10} having from 1 to about 8 member atoms;

(e) Y is $(CH_2)_n$; n being an integer from 0 to about 3;

(f) Z is carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic; and

any optical isomer, diasteroemer, enantiomer of the above structure, or a pharmaceutically-acceptable salt, or biohydrolyzable amide, ester, or imide thereof.

17. The method of claim **16** wherein said bone disorder is osteoporosis.

18. The method of claim **17** wherein said bone disorder is post-menopausal.

19. The method of claim **17** wherein said bone disorder is cortico-steroid induced.

20. The method of claim **16** wherein said bone disorder is osteopenia.

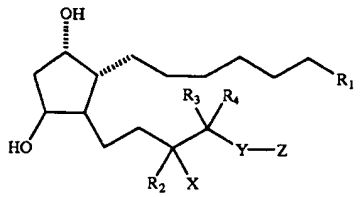
21. The method of claim **16** wherein said bone disorder is a bone fracture.

22. The method of claim **16** wherein said compound is administered orally.

23. The method of claim **16** wherein said compound is administered transdermally.

24. The method of claim **16** wherein said compound is administered intranasally.

25. A method of treating glaucoma, said method comprising administering to a human or other animal a safe and effective amount of a compound according to the structure:



wherein

(a) R_1 is CO_2H, C(O)NHOH, CO_2R_5, CH_2OH, $S(O)_2R_5$, $C(O)NHR_5$, $C(O)NHS(O)_2R_5$, or tetrazole; wherein R_5 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(b) R_2 is H or lower alkyl;

(c) X is NR_6R_7, OR_8, SR_9, $S(O)R_9$, $S(O)_2R_9$, or F; wherein R_6, R_7, and R_8 are independently selected from the group consisting of H, acyl, alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, and heteroaromatic ring; and wherein R_9 is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring;

(d) R_3 and R_4 are independently H, CH_3, C_2H_5, OR_{10}, SR_{10}, or OH, except that both R_3 and R_4 are not OH; wherein R_{10} is alkyl, heteroalkyl, carbocyclic aliphatic ring, heterocyclic aliphatic ring, aromatic ring, or heteroaromatic ring, R_{10} having from 1 to about 8 member atoms;

(e) Y is $(CH_2)_n$; n being an integer from 0 to about 3;

(f) Z is carbocyclic aliphatic ring, heterocyclic aliphatic ring, heteroaromatic ring, substituted phenyl, or substituted or unsubstituted napthyl when n is 0, 2, or 3; and Z is carbocyclic aliphatic ring, heterocyclic aliphatic ring, substituted phenyl, or substituted or unsubstituted napthyl when n is 1; and

any optical isomer, diasteroemer, enantiomer of the above structure, or a pharmaceutically-acceptable salt, or biohydrolyzable amide, ester, or imide thereof.

26. The method of claim **25** wherein said compound is administered topically.

* * * * *

US006096697A

United States Patent [19]

Wells

[11] Patent Number: 6,096,697

[45] Date of Patent: *Aug. 1, 2000

[54] **PERSONAL CLEANSING COMPOSITIONS PROVIDING IMPROVED HAIR AND SKIN CONDITIONING**

[75] Inventor: **Robert Lee Wells**, Cincinnati, Ohio

[73] Assignee: **The Procter & Gamble Company**, Cincinnati, Ohio

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **09/082,877**

[22] Filed: **May 21, 1998**

Related U.S. Application Data

[63] Continuation of application No. 08/786,578, Jan. 21, 1997, Pat. No. 5,785,979.

[51] **Int. Cl.**[7] **A61K 7/075**; A61K 7/50; C11D 17/00; C11D 3/38

[52] **U.S. Cl.** **510/127**; 510/122; 510/159; 510/426; 510/428; 510/472

[58] **Field of Search** 510/130, 156, 510/472, 159, 426, 428, 119, 122, 127

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[57] **ABSTRACT**

The compositions of the present invention relate to improved personal cleansing compositions comprising a surfactant system, from above about 0.1% to below 1% of a nonionic or anionic water soluble polymer, a phase separation initiator and water. These compositions provide improved lathering and conditioning benefits.

20 Claims, No Drawings

PERSONAL CLEANSING COMPOSITIONS PROVIDING IMPROVED HAIR AND SKIN CONDITIONING

This application is a continuation of application Ser. No. 08/786,578, filed Jan. 21, 1997, now U.S. Pat. No. 5,785,979.

FIELD OF THE INVENTION

The present invention relates personal cleansing compositions comprising a cleansing component together with a conditioning component.

BACKGROUND OF THE INVENTION

Human hair becomes soiled due to its contact with the surrounding atmosphere and, to a greater extent, from sebum secreted by the head. The build-up of the sebum causes the hair to have a dirty feel and an unattractive appearance. The soiling of the hair necessitates it being shampooed with frequent regularity.

Shampooing the hair cleans by removing excess soil and sebum. However, the shampooing process has disadvantages in that the hair is left in a wet, tangled and generally unmanageable state. Shampooing can also result in the hair becoming dry or "frizzy", and a loss of luster, due to removal of natural oils or other hair moisturizing materials. After shampooing, the hair can also suffer from a loss of "softness" perceived by the user upon drying. The hair can also suffer from increased levels of static upon drying after shampooing. This can interfere with combing and can result in fly-away hair. A variety of approaches have been developed to alleviate the after-shampoo problems. These range from the inclusion of hair conditioning aids in shampoos to post-shampoo application of hair conditioners, i.e., hair rinses. Hair rinses are generally liquid in nature and must be applied in a separate step following the shampooing, left on the hair for a length of time, and rinsed with fresh water. This, of course, is time consuming and is not as convenient as shampoos containing both cleaning and hair conditioning ingredients.

While a wide variety of shampoos have been disclosed which contain conditioning aids, they have not been totally satisfactory for a variety of reasons. Cationic conditioning agents are highly desirable for use in hair conditioning due to their abilities to control static, improve wet detangling, and provide a silky wet hair feel to the user. One problem which has been encountered in shampoos relates to compatibility problems between good cleaning anionic surfactants and the many conventional cationic agents which historically have been used as conditioning agents. Efforts have been made to minimize adverse interaction through the use of alternate surfactants and improved cationic conditioning agents. Cationic surfactants which provide good overall conditioning in hair rinse products, in general, tend to complex with anionic cleaning surfactants and provide poor conditioning in a shampoo context. In particular, the use of soluble cationic surfactants that form soluble ionic complexes do not deposit well on the hair. Soluble cationic surfactants that form insoluble ionic complexes deposit on the hair but do not provide good hair conditioning benefits, and tend to cause the hair to have a dirty, coated feel. The use of insoluble cationic surfactants, e.g., tricetyl methyl ammonium chloride, can provide excellent anti-static benefits but do not otherwise provide good overall conditioning. Many cationic polymers tend to build up on the hair, resulting in an undesirable, "unclean" coated feel. Cationic polymers therefore, conventionally, are preferably used at limited levels to minimize this problem. This, however, can limit the overall conditioning benefits that are obtained. Additionally, cationic conditioning agents commonly do not provide optimal overall conditioning benefits, particularly in the area of "softness", especially when delivered as an ingredient in a shampoo composition.

Materials which can provide increased softness are nonionic silicones. Silicones in shampoo compositions have been disclosed in a number of different publications. Such publications include U.S. Pat. No. 2,826,551, Geen, issued Mar. 11, 1958; U.S. Pat. No. 3,964,500, Drakoff, issued Jun. 22, 1976; U.S. Pat. No. 4,364,837, Pader, issued Dec. 21, 1982; and British Patent 849,433, Woolston, issued Sep. 28, 1960. While these patents disclose silicone containing compositions, they also did not provide a totally satisfactory product in that it was difficult to maintain the silicone well dispersed and suspended in the product. Recently, stable, insoluble silicone-containing hair conditioning shampoo compositions have been described in U.S. Pat. No. 4,741,855, Grote and Russell, issued May 3, 1988 and U.S. Pat. No. 4,788,066, Bolich and Williams, issued Nov. 29, 1988. These shampoo compositions can deliver excellent overall conditioning benefits to the hair while maintaining excellent cleaning performance, even with the use of anionic detersive surfactants, for a wide variety of hair types.

More recently, improved conditioning shampoos were provided in U.S. Ser. No. 07/622,699, Robert L. Wells, filed Dec. 5, 1990, now abandoned, and its continuation application Ser. No. 07/778,765, filed Oct. 21, 1991, wherein shampoos containing anionic surfactant, dispersed, insoluble silicone, and certain relatively low ionic strength cationic polymers (greater than about 0.4 meq./gm) were disclosed. These compositions provide excellent hair cleaning and conditioning to a wide variety of hair types, especially including improved conditioning to hair damaged by color treatments, bleaching, permanents, etc.

Japanese Patent Application, Laid Open No. 56-72095, Jun. 16, 1981, Hirota et al. (Kao Soap Corp.) also discloses shampoo containing cationic polymer and silicone conditioning agents. Still other patent publications relating to shampoos with cationic agents and silicone include EPO Application Publication 0 413 417, published Feb. 20, 1991, Hartnett et al.

Another approach to providing hair conditioning benefits to shampoo compositions has been to use materials which are oily to the touch. These materials provide improved luster and shine to the hair. Oily materials have also been combined with cationic materials in the shampoo formulations. Japanese Patent Application Showa 53-35902, laid open October 6, 1979 (Showa 54-129135), N. Uchino (Lion Yushi Co.), discloses hair treatment compositions containing cationic polymer, fatty acid salt, and at least 10% of an oily component for use before or after shampooing. Suitable oily components are hydrocarbons, higher alcohols, fatty acid esters, glycerides, and fatty acids. Japanese Patent Application 62 [1987]-327266, filed Dec. 25, 1987, published Jul. 4, 1989, laid open No. HEI 1[1987]-168612, Horie et al., discloses detergent compositions containing cationic surfactant and/or cationic polymer, anionic surfactant, and specific esters of the formula RCOOR' wherein R and R' are straight or branched chain alkyls.

In spite of these attempts to provide optimal combinations of cleaning ability and hair conditioning, there remains a need for personal cleansing compositions providing improved lathering and conditioning benefits. The present

inventor has found that compositions combining certain nonionic or anionic polymers in combination with surfactants and a minimum level of a phase separation initiator form stable aqueous emulsions—wherein the emulsion comprises aqueous polymer phase droplets suspended in an aqueous surfactant phase. The dispersed, concentrated polymer phase provides improved hair and skin conditioning without sacrificing clean feel. These compositions can be made into any of a number of conventional forms including, but not limited to, conditioning shampoos, foams, mousses, gels, lotions, sprays and the like.

In addition to the afore-mentioned hair care benefits, it has been found that the nonionic or anionic polymer and surfactant system emulsion of the present invention is also useful for incorporation into a wide variety of personal skin cleansing compositions or used in conjunction with lathering instruments. These compositions provide a skin conditioning component which is more easily and uniformly deposited upon the skin and feel good upon the skin. Such compositions include liquid soaps, shower gels, lotions and the like. Suitable lathering instruments include nonwoven substrates, woven substrates, hydroentangled substrates, air entangled substrates, natural sponges, synthetic sponges, polymeric netted meshes, and the like.

Accordingly, it is an object of this invention to provide personal cleansing compositions providing improved lathering and conditioning.

Another object of the present invention is to provide improved personal cleansing compositions comprising a nonionic or anionic polymer, a surfactant system and a minimum amount of a phase separation initiator.

One other object of the present invention is to provide personal cleaning compositions which exist as an emulsion comprising a polymer concentrated aqueous phase in an aqueous surfactant phase.

Still another object of the present invention is to provide personal cleansing compositions which can comprise lower or reduced levels of surfactant.

Another object of the present invention is to provide all in one shampoo plus conditioner compositions which can provide excellent cleaning performance and improved levels of conditioning while minimizing any adverse side effects associated with build-up due to the use of excess conditioning agent.

It is also an object of this invention to provide a method for cleaning and conditioning the hair and skin which can provide excellent cleaning in combination with improved conditioning.

These and other objects will become readily apparent from the detailed description which follows.

SUMMARY OF THE INVENTION

The present invention relates to personal cleansing compositions in the form of a stable aqueous emulsion, comprising:

a.) from about 4% to about 50%, by weight, of an aqueous surfactant system;

b.) from above about 0.1% to less than 1.0%, by weight, of a nonionic or anionic, water soluble polymer;

c.) from about 0.1% to about 5%, by weight, of a phase separation initiator selected from the group consisting of electrolytes, amphiphiles and mixtures thereof; and

e.) from about 50% to about 95%, by weight, of water wherein said polymer forms visually distinct aqueous droplets in the aqueous surfactant system.

The present invention further relates to methods of using the personal cleansing compositions.

DETAILED DESCRIPTION OF THE INVENTION

The personal cleansing compositions of the present invention can comprise, consist of, or consist essentially of the essential elements and limitations of the invention described herein, as well any of the additional or optional ingredients, components, or limitations described herein.

All percentages, parts and ratios are based upon the total weight of the personal cleansing compositions of the present invention, unless otherwise specified. All such weights as they pertain to listed ingredients are based on the active level and, therefore, do not include carriers or by-products that may be included in commercially available materials, unless otherwise specified.

The term "phase separation", as used herein, means the formation of two thermodynamically stable liquid phases which exist, not as distinct bulk layers, but as a stable emulsion comprising droplets of one phase dispersed in another phase.

The term "visually distinct", as used herein, refers to droplets or droplet phases suspended in a continuous phase such that, optically, the droplets or droplet phases are visually separate and distinct from the continuous phase when viewed by the unaided eye.

As used herein, the term "water soluble" refers to any material that is sufficiently soluble in water to form a substantially clear solution to the naked eye at a concentrations of 1.0% or more by weight of the material in the water at 25° C. Conversely, the term "water insoluble" refers to all materials that are not sufficiently soluble in water to form a substantially clear solution to the naked eye at a concentration of about 1.0% or more by weight of the insoluble material in water at 25° C.

The personal cleansing compositions of the present invention, including the essential and optional components thereof, are described in detail hereinafter.

Essential Components

Surfactant System

The compositions of the present invention comprise as an essential component a suitable surfactant system.

The surfactant system of the present invention is preferably present in the personal cleansing compositions at a level of from about 4% to about 50%, more preferably from about 4% to about 40%, still more preferably from about 4% to about 30%, even more preferably from about 5% to about 20% and most preferably from about 6% to about 16%. It should be recognized, however, that the concentration of the surfactant system may vary with the cleaning or lather performance desired, the surfactants incorporated into the surfactant system, the desired product concentration, the presence of other components in the composition, and other factors well known in the art.

The surfactant system of the present invention comprises primary detersive surfactants selected from the group consisting of anionic surfactants, amphoteric surfactants and mixtures thereot as well as additional detersive surfactants selected from the group consisting of nonionic surfactants, cationic surfactants or mixtures thereof. The purpose of the detersive surfactant is to provide cleaning performance to the composition. Amphoteric surfactant components useful in the present composition include those known to be useful

in personal cleansing compositions, and which, preferably, contain a group that is anionic at the pH of the compositions of the present invention. The concentration of such surfactant components in the surfactant system of the present invention preferably ranges from about 0.5% to about 20%, more preferably from about 1% to about 10%, and most preferably from about 2% to about 7% by weight of the surfactant system. Examples of amphoteric surfactants suitable for use in the personal cleansing composition herein are described in U.S. Pat. No. 5,104,646 (Bolich Jr. et al.), U.S. Pat. No. 5,106,609 (Bolich Jr. et al.), which descriptions are incorporated herein by reference. Examples of amphoteric detersive surfactants which can be used in the compositions of the present invention are those which are broadly described as derivatives of aliphatic secondary and tertiary amines in which the aliphatic radical can be straight or branched chain and wherein one of the aliphatic substituents contains from about 8 to about 18 carbon atoms and one contains an anionic water solubilizing group, e.g., carboxy, sulfonate, sulfate, phosphate, or phosphonate. Examples of compounds falling within this definition are sodium 3-dodecyl-aminopropionate, sodium 3-dodecylaminopropane sulfonate, sodium lauroamphoacetate, N-alkyltaurines such as the one prepared by reacting dodecylamine with sodium isethionate according to the teaching of U.S. Pat. No. 2,658,072, N-higher alkyl aspartic acids such as those produced according to the teaching of U.S. Pat. No. 2,438,091, and the products sold under the trade name "MIRANOL"™ and described in U.S. Pat. No. 2,528,378.

Other amphoterics, sometimes classified as zwitterionics, such as betaines can also be used in the present invention. Such zwitterionics are considered as amphoterics in the present invention where the zwitterionic has an attached group that is anionic at the pH of the composition. Examples of betaines useful herein include the high alkyl betaines, such as coco dimethyl carboxymethyl betaine, cocoamidopropyl betaine, cocobetaine, lauryl amidopropyl betaine, oleyl betaine, lauryl dimethyl carboxymethyl betaine, lauryl dimethyl alphacarboxyethyl betaine, cetyl dimethyl carboxymethyl betaine, lauryl bis-(2-hydroxyethyl) carboxymethyl betaine, stearyl bis-(2-hydroxypropyl) carboxymethyl betaine, oleyl dimethyl gamma-carboxypropyl betaine, and lauryl bis-(2-hydroxypropyl)alpha-carboxyethyl betaine. The sulfobetaines may be represented by coco dimethyl sulfopropyl betaine, stearyl dimethyl sulfopropyl betaine, lauryl dimethyl sulfoethyl betaine, lauryl bis-(2-hydroxyethyl) sulfopropyl betaine and the like; amidobetaines and amidosulfobetaines, wherein the $RCONH(CH_2)_3$ radical is attached to the nitrogen atom of the betaine are also useful in this invention. Most preferred for use herein is cocoamidopropyl betaine.

Suitable anionic surfactants include alkyl sulfate, alkyl ethoxylated sulfate, or a mixture thereof. These materials have the respective formulae (I) $ROSO_3M$ and (II) $RO(C_2H_4O)_xSO_3M$, wherein R is alkyl or alkenyl of from about 8 to about 30 carbon atoms, x is 1 to 10, and M is H or a salt-forming cation such as ammonium, alkanolamine containing C_1–C_3 alkyl groups such as triethanolamine, and monovalent and polyvalent metals such as the alkaline and alkaline earth metals. Preferred metals include sodium, potassium, magnesium, and calcium. The cation M, of the anionic surfactant should preferably be chosen such that the anionic surfactant component is water soluble. Solubility of anionic surfactants, in general, will depend upon the particular anionic surfactants and cations chosen. As an aid to determining appropriate mixtures of anionic surfactants, the anionic surfactants should be chosen such that the Krafft temperature is about 15° C. or less, preferably about 10° C. or less, more preferably about 0° C. or less. It is also preferred that the anionic surfactant be soluble in the composition hereof.

Preferably, R has from about 10 to about 18 carbon atoms in both the alkyl and alkyl ethoxylated sulfates. The alkyl ethoxylated sulfates are typically made as condensation products of ethylene oxide and monohydric alcohols having from about 8 to about 24 carbon atoms. The alcohols can be derived from fats, e.g., coconut oil, palm kernel oil, or tallow, or can be synthetic. Such alcohols are preferably reacted with about 1 to about 10, more preferably from about 1 to about 4, most preferably from about 2 to about 3.5, molar proportions of ethylene oxide and the resulting mixture of molecular species having, for example, an average of 3 moles of ethylene oxide per mole of alcohol, is sulfated and neutralized.

Specific examples of alkyl ether sulfates which may be used in the present invention are sodium and ammonium salts of coconut alkyl triethylene glycol ether sulfate; tallow alkyl triethylene glycol ether sulfate, and tallow alkyl hexaoxyethylene sulfate. Highly preferred alkyl ether sulfates are those comprising a mixture of individual compounds, said mixture having an average alkyl chain length of from about 12 to about 16 carbon atoms and an average degree of ethoxylation of from about 1 to about 4 moles of ethylene oxide.

The sulfate surfactant is preferably comprised of a combination of ethoxylated and nonethoxylated sulfates. Alkyl sulfates can provide excellent cleaning and lather performance. Alkyl ethoxylated sulfates can provide excellent cleaning performance and are mild to the skin.

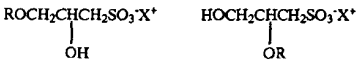
Other suitable anionic detersive surfactants include, but are not limited to water-soluble salts of organic, sulfuric acid reaction products of the general formula $[R_1—SO_3—M]$ where R_1 is selected from the group consisting of a straight or branched chain, saturated aliphatic hydrocarbon radical having from about 8 to about 24, preferably about 10 to about 18, carbon atoms; and M is a cation such as ammonium, alkanolamines, such as triethanolamine, monovalent metals, such as sodium and potassium, and polyvalent metal cations, such as magnesium, and calcium. The cation M, of the anionic detersive surfactant should be chosen such that the detersive surfactant component is water soluble. Solubility will depend upon the particular anionic detersive surfactants and cations chosen. Examples of such detersive surfactants are the salts of an organic sulfuric acid reaction product of a hydrocarbon of the methane series, including iso-, neo-, and n-paraffins, having about 8 to about 24 carbon atoms, preferably about 10 to about 18 carbon atoms and a sulfonating agent, e.g., SO_3, H_2SO_4, obtained according to known sulfonation methods, including bleaching and hydrolysis. Preferred are alkali metal and ammonium sulfonated C_{10-18} n-paraffins.

Another class of anionic detersive surfactants suitable for use in the present invention are the reaction products of fatty acids esterified with isethionic acid and neutralized with sodium hydroxide where, for example, the fatty acids are derived from coconut oil or palm kernal oil; sodium, ammonium, tetraethylammonium or potassium salts of fatty acid amides of methyl tauride in which the fatty acids, for example, are derived from coconut oil or palm kernal oil. Other similar anionic surfactants are described in U.S. Pat. No. 2,486,921; U.S. Pat. No. 2,486,922; and U.S. Pat. No. 2,396,278, which descriptions are incorporated herein by reference.

Other anionic detersive surfactants suitable for use in the present invention are the succinnates, examples of which include disodium N-octadecylsulfosuccinnate; disodium lauryl sulfosuccinate; diammonium lauryl sulfosuccinate; tetrasodium N-(1,2-dicarboxyethyl)-N-octadecylsulfosuccinnate; diamyl ester of sodium sulfosuccinic acid; dihexyl ester of sodium sulfosuccinic acid; dioctyl esters of sodium sulfosuccinic acid.

Other suitable anionic detersive surfactants include alkyl glyceryl ether sulfonate surfactants (also referred to herein as an "AGS" surfactant), derivatives thereof and salts thereof. These AGS surfactants are derived from an alkyl glyceryl ether containing a sulfonate or sulfonate salt group. These compounds generally can be described as an alkyl monoether of glycerol that also contains a sulfonate group.

These AGS surfactants can be described as generally conforming to the following structures:



wherein R is a saturated or unsaturated straight chain, branched chain, or cyclic alkyl group having from about 10 to about 18 carbon atoms, preferably from about 11 to about 16 carbon atoms, and most preferably from about 12 to about 14 carbon atoms, and X is a cation selected from the group consisting of ammonium; mono-alkylsubstituted ammonium; di-alkylsubstituted ammonium; tri-alkylsubstituted ammonium; tetra-alkylsubstituted ammonium; alkali metal; alkaline metal; and mixtures thereof. More preferably, the alkyl radicals, R in the above formulas, are saturated and straight chain.

Without being limited by theory, it is believed that the distribution of alkyl chain lengths in the AGS surfactant has some effect on the character of the overall cleansing composition. A satisfactory distribution can be achieved in a commercially practicable way by using fatty alcohols derived from coconut oil and tallow. An equivalent distribution of alkyl chain lengths can be achieved using other starting materials. In the preparation of the coconut fatty alcohols used to provide the alkyl group of the AGS, preferably the middle cut of the coconut oil is taken. The higher boiling cut can be retained with the middle cut coconut oils if desired. In the preparation of the tallow fatty alcohols, a hydrogenation step is included to insure that they are substantially saturated.

The preferred AGS compounds are those where the alkyl group is derived from at least about 50% from alcohols of about 10 to about 18 carbons, having mainly monoglyceryl radicals present, with less than about 30% of diglyceryl radicals present. The AGS used in the Examples described below contains about 15% of diglyceryl ether sulfonates, and is preferred because of the ease of manufacturing this material. The term "AGS" is intended to include monoglyceryl, diglyceryl, and traces of the higher glyceryl compounds. Small amounts, that is less than about 3% total, of triglyceryl ether sulfonates and tetraglyceryl ether sulfonates can be present. Also included are AGS's derived from glyceryl ethers having branched or mixed branched and straight chain lengths that emulate the straight chain lengths.

The AGS surfactants useful in the present invention are more fully described in U.S. Pat. No. No. 2,979,465, to Parran et al., issued Apr. 11, 1961; U.S. Pat. No. 3,179,599, to Eaton et al., issued Apr. 20, 1965; British Patent No. 848,224, published Sep. 14, 1960; British Patent No. 791, 415, published Mar. 5, 1958; U.S. Pat. No. 5,322,643, to Schwartz et al., issued Jun. 21, 1994; and U.S. Pat. No. 5,084,212, to Farris et al. issued Jan. 28, 1992; which are all hereby incorporated herein by reference in their entirety. These references also disclose various cleansing products in which the AGS surfactant of this invention can be used.

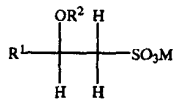
Still other suitable anionic detersive surfactants include olefin sulfonates having about 10 to about 24 carbon atoms. The term "olefin sulfonates" is used herein to mean compounds which can be produced by the sulfonation of alpha-olefins by means of uncomplexed sulfur trioxide, followed by neutralization of the acid reaction mixture in conditions such that any sulfones which have been formed in the reaction are hydrolyzed to give the corresponding hydroxy-alkanesulfonates. The sulfur trioxide can be liquid or gaseous, and is usually, but not necessarily, diluted by inert diluents, for example by liquid SO_2, chlorinated hydrocarbons, etc., when used in the liquid form, or by air, nitrogen, gaseous SO_2, etc., when used in the gaseous form.

The alpha-olefins from which the olefin sulfonates are derived are mono-olefins having about 10 to about 24 carbon atoms, preferably about 12 to about 16 carbon atoms. Preferably, they are straight chain olefins.

In addition to the true alkene sulfonates and a proportion of hydroxy-alkanesulfonates, the olefin sulfonates can contain minor amounts of other materials, such as alkene disulfonates depending upon the reaction conditions, proportion of reactants, the nature of the starting olefins and impurities in the olefin stock and side reactions during the sulfonation process.

A specific alpha-olefin sulfonate mixture of the above type is described more fully in the U.S. Pat. No. 3,332,880, which description is incorporated herein by reference.

Another class of anionic detersive surfactants suitable for use in the present invention are the beta-alkyloxy alkane sulfonates. These compounds have the following formula:



where R^1 is a straight chain alkyl group having from about 6 to about 20 carbon atoms, R^2 is a lower alkyl group preferably having from about 1 to about 3 carbon atoms, and M is a water-soluble cation as hereinbefore described.

Preferred additional anionic detersive surfactants for use in the present invention include alkyl glyceryl ether sulfonate, ammonium lauryl sulfate, ammonium laureth sulfate, triethylamine lauryl sulfate, triethylamine laureth sulfate, triethanolamine lauryl sulfate, triethanolamine laureth sulfate, monoethanolamine lauryl sulfate, monoethanolamine laureth sulfate, diethanolamine lauryl sulfate, diethanolamine laureth sulfate, lauric monoglyceride sodium sulfate, sodium lauryl sulfate, sodium laureth sulfate, potassium lauryl sulfate, potassium laureth sulfate, sodium lauryl sarcosinate, sodium lauroyl sarcosinate, lauryl sarcosine, cocoyl sarcosine, ammonium cocoyl sulfate, ammonium lauroyl sulfate, sodium cocoyl sulfate, sodium lauroyl sulfate, potassium cocoyl sulfate, potassium lauryl sulfate, triethanolamine lauryl sulfate, triethanolamine lauryl sulfate, monoethanolamine cocoyl sulfate, monoethanolamine lauryl sulfate, sodium tridecyl benzene sulfonate, sodium dodecyl benzene sulfonate, and combinations thereof.

The anionic detersive surfactants are preferably present in the surfactant system of the present invention at a concen-

tration level of from about 3% to about 20%, more preferably from about 4% to about 17%, most preferably from about 6% to about 14%.

Another class of anionic surfactants is fatty acid soaps. Though useful to the present invention, high concentrations of these surfactants in the presence of hard water tend to result in significant buildup on the hair and skin, adversely affecting cleansing and hair and skin feel. Accordingly, if added to the compositions of the present invention, the level of the fatty acid soaps is preferably incorporated at concentration levels of less than about 3%, more preferably less than about 1%.

The surfactant system of the present invention may also include nonionic surfactants, cationic surfactants, and combinations thereof. Suitable classes of nonionic surfactants include:

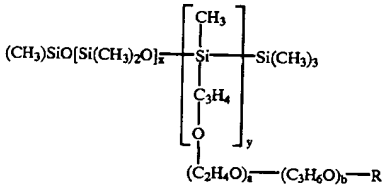
1. The polyethylene oxide condensates of alkyl phenols, e.g., the condensation products of alkyl phenols having an alkyl group containing from about 6 to about 12 carbon atoms in either a straight chain or branched chain configuration, with ethylene oxide, the said ethylene oxide being present in amounts equal to from about 10 to about 60 moles of ethylene oxide per mole of alkyl phenol. The alkyl substituent in such compounds may be derived from polymerized propylene, diisobutylene, octane, or nonane, for example.
2. Those derived from the condensation of ethylene oxide with the product resulting from the reaction of propylene oxide and ethylene diamine products which may be varied in composition depending upon the balance between the hydrophobic and hydrophilic elements which is desired. For example, compounds containing from about 40% to about 80% polyoxyethylene by weight and having a molecular weight of from about 5,000 to about 11,000 resulting from the reaction of ethylene oxide groups with a hydrophobic base constituted of the reaction product of ethylene diamine and excess propylene oxide, said base having a molecular weight of the order of about 2,500 to about 3,000, are satisfactory.
3. The condensation product of aliphatic alcohols having from about 8 to about 18 carbon atoms, in either straight chain or branched chain configuration, with ethylene oxide, e.g., a coconut alcohol ethylene oxide condensate having from about 10 to about 30 moles of ethylene oxide per mole of coconut alcohol, the coconut alcohol fraction having from about 10 to about 14 carbon atoms.
4. Long chain tertiary amine oxides corresponding to the following general formula:

$$R_1R_2R_3N\rightarrow O$$

wherein R_1 contains an alkyl, alkenyl or monohydroxy alkyl radical of from about 8 to about 18 carbon atoms, from 0 to about 10 ethylene oxide moieties, and from 0 to about 1 glyceryl moiety, and R_2 and R_3 contain from about 1 to about 3 carbon atoms and from 0 to about 1 hydroxy group, e.g., methyl, ethyl, propyl, hydroxyethyl, or hydroxypropyl radicals. The arrow in the formula is a conventional representation of a semipolar bond. Examples of amine oxides suitable for use in this invention include dimethyl-dodecylamine oxide, oleyldi(2-hydroxyethyl) amine oxide, dimethyloctylamine oxide, dimethyl-decylamine oxide, dimethyltetradecylamine oxide, 3,6,9-trioxaheptadecyldiethylamine oxide, di(2-hydroxyethyl)-tetradecylamine oxide, 2-dodecoxyethyldimethylamine oxide, 3-dodecoxy-2-hydroxypropyldi(3-hydroxypropyl) amine oxide, dimethylhexadecylamine oxide.
5. Long chain tertiary phosphine oxides corresponding to the following general formula:

$$RR'R''P\rightarrow O$$

wherein R contains an alkyl, alkenyl or monohydroxyalkyl radical ranging from about 8 to about 18 carbon atoms in chain length, from 0 to about 10 ethylene oxide moieties and from 0 to about 1 glyceryl moiety and R' and R" are each alkyl or monohydroxyalkyl groups containing from about 1 to about 3 carbon atoms. The arrow in the formula is a conventional representation of a semipolar bond. Examples of suitable phosphine oxides are: dodecyldimethylphosphine oxide, tetradecyldimethylphosphine oxide, tetradecylmethylethylphosphine oxide, 3,6,9,-trioxaoctadecyldimethylphosphine oxide, cetyldimethylphosphine oxide, 3-dodecoxy-2-hydroxypropyldi(2-hydroxyethyl) phosphine oxide, stearyldimethylphosphine oxide, cetylethylpropylphosphine oxide, oleyldiethylphosphine oxide, dodecyldiethylphosphine oxide, tetradecyldiethylphosphine oxide, dodecyldipropylphosphine oxide, dodecyldi(hydroxymethyl)phosphine oxide, dodecyldi(2-hydroxyethyl)phosphine oxide, tetradecylmethyl-2-hydroxypropylphosphine oxide, oleydimethylphosphine oxide, 2-hydroxydodecyldimethylphosphine oxide.
6. Long chain dialkyl sulfoxides containing one short chain alkyl or hydroxy alkyl radical of from about 1 to about 3 carbon atoms (usually methyl) and one long hydrophobic chain which include alkyl, alkenyl, hydroxy alkyl, or keto alkyl radicals containing from about 8 to about 20 carbon atoms, from 0 to about 10 ethylene oxide moieties and from 0 to about 1 glyceryl moiety. Examples include: octadecyl methyl sulfoxide, 2-ketotridecyl methyl sulfoxide, 3,6,9,-trixaoctadecyl 2-hydroxyethyl sulfoxide, dodecyl methyl sulfoxide, oleyl 3-hydroxypropyl sulfoxide, tetradecyl methyl sulfoxide, 3-methoxytridecyl methyl sulfoxide, 3-hydroxytridecyl methyl sulfoxide, 3-hydroxy-4-dodecoxybutyl methyl sulfoxide.
7. Polyalkylene oxide modified dimethylpolysiloxanes, also known as dimethicone copolyols. These materials include the polyalkylene oxide modified dimethylpolysiloxanes of the following formulae:



and

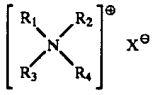
$$R'—Si—[[O—Si—(CH_3)_2]_x(OC_2H_4)_a—(OC_3H_6)_b—OR'']_3$$

wherein R is hydrogen, an alkyl group having from 1 to about 12 carbon atoms, an alkoxy group having from 1 to

about 6 carbon atoms or a hydroxyl group; R' and R" are alkyl groups having from 1 to about 12 carbon atoms; x is an integer of from 1 to 100, preferably from 20 to 30; y is an integer of 1 to 20, preferably from 2 to 10; and a and b are integers of from 0 to 50, preferably from 20 to 30. Dimethicone copolyols among those useful herein are disclosed in the following patent documents, all incorporated by reference herein: U.S. Pat. No. 4,122,029, Gee et al., issued Oct. 24, 1978; U.S. Pat. No. 4,265,878, Keil, issued May 5, 1981; and U.S. Pat. No. 4,421,769, Dixon et al., issued Dec. 20, 1983. Commercially available dimethicone copolyols, useful herein, include Silwet Surface Active Copolymers (manufactured by the Union Carbide Corporation); Dow Corning Silicone Surfactants (manufactured by the Dow Corning Corporation); Silicone Copolymer F-754 (manufactured by SWS Silicones Corp.); and Rhodorsil 70646 Fluid (manufactured by Rhone Poulenc, Inc.).

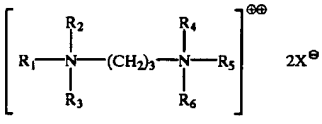
Cationic surfactants are also useful in compositions of the present invention and typically contain amino or quaternary ammonium hydrophilic moieties which are positively charged when dissolved in the aqueous composition of the present invention. Cationic surfactants among those useful herein are disclosed in the following documents, all incorporated by reference herein: M.C. Publishing Co., *McCutcheon's, Detergents & Emulsifiers,* (North American edition 1989); Schwartz, et al., *Surface Active Agents, Their Chemistry and Technology.* New York: Interscience Publishers, 1949; U.S. Pat. No. 3,155,591, Hilfer, issued Nov. 3, 1964; U.S. Pat. No. 3,929,678, Laughlin et al., issued Dec. 30, 1975; U.S. Pat. No. 3,959,461, Bailey et al., issued May 25, 1976; and U.S. Pat. No. 4,387,090, Bolich, Jr., issued Jun. 7, 1983. If included in the compositions of the present invention, the cationic surfactant must not interfere with the in-use performance and end-benefits of the personal cleansing composition.

Among the quaternary ammonium-containing cationic surfactant materials useful herein are those of the general formula:



wherein R_1–R_4 are independently an aliphatic group of from about 1 to about 22 carbon atoms, or an aromatic, alkoxy, polyoxyalkylene, alkylamido, hydroxyalkyl, aryl or alkylaryl group having from about 12 to about 22 carbon atoms; and X is an anion selected from halogen, acetate, phosphate, nitrate and alkylsulfate radicals. The aliphatic groups may contain, in addition to carbon and hydrogen atoms, ether linkages, and other groups such as amino groups.

Other quaternary ammonium salts useful herein have the formula:



wherein R_1 is an aliphatic group having from about 16 to about 22 carbon atoms, R_2, R_3, R_4, R_5, and R_6 are selected from hydrogen and alkyl having from about 1 to about 4 carbon atoms, and X is an ion selected from halogen, acetate, phosphate, nitrate and alkyl sulfate radicals. Such quaternary ammonium salts include tallow propane diammonium dichloride.

Preferred quaternary ammonium salts include monoalkyltrimethylammonium chlorides and dialkyldimethylammonium chlorides and trialkyl methyl ammonium chlorides, wherein at least one of the alkyl groups have from about 12 to about 22 carbon atoms and are derived from long-chain fatty acids, such as hydrogenated tallow fatty acid (tallow fatty acids yield quaternary compounds wherein the long chain alkyl groups are predominately from 16 to 18 carbon atoms). Examples of quaternary ammonium salts useful in the present invention include stearyl trimethyl ammonium chloride, ditallowdimethyl ammonium chloride, ditallowdimethyl ammonium methyl sulfate, dihexadecyl dimethyl ammonium chloride, di(hydrogenated tallow) dimethyl ammonium chloride, dioctadecyl dimethyl ammonium chloride, dieicosyl dimethyl ammonium chloride, didocosyl dimethyl ammonium chloride, di(hydrogenated tallow) dimethyl ammonium acetate, dihexadecyl dimethyl ammonium chloride, dihexadecyl dimethyl ammonium acetate, ditallow dipropyl ammonium phosphate, ditallow dimethyl ammonium nitrate, di(coconutalkyl) dimethyl ammonium chloride, and stearyl dimethyl benzyl ammonium chloride, ditallow dimethyl ammonium chloride, dicetyl dimethyl ammonium chloride, stearyl dimethyl benzyl ammonium chloride and cetyl trimethyl ammonium chloride are preferred quaternary ammonium salts useful herein. Di-(hydrogenated tallow) dimethyl ammonium chloride and tricetyl methyl ammonium chloride are particularly preferred quaternary ammonium salts. These materials also provide anti-static benefits to shampoo embodiments of the present invention.

Other surfactants known in the art for use in hair or personal cleansing products may be used in the surfactant system of the present invention, provided that the surfactant is also chemically and physically compatible with the essential components of the present invention, or does not otherwise unduly impair product performance, aesthetics or stability. Preferred for use in the surfactant system of the present invention are anionic and/or amphoteric surfactants.

Though useful to the compositions of the present invention, nonionic or cationic surfactants tend to reduce the lathering properties of soap and shampoo compositions. To maintain adequate lathering profiles, nonionic or cationic surfactants are preferably present at low concentrations. Generally, the surfactant system of the present invention will contain less than 3%, more preferably less than 1% of the nonionic and cationic surfactant.

Nonionic or Anionic Water-Soluble Polymer

Another essential component of the present invention is a nonionic or anionic water-soluble polymer. Suitable nonionic polymers include such water soluble polymers as cellulose ethers (e.g., hydroxybutyl methylcellulose, hydroxypropylcellulose, hydroxypropyl methylcellulose, ethylhydroxy ethylcellulose and hydroxyethylcellulose), propylene glycol alginates, polyacrylamide, poly(ethylene oxide), polyvinyl alcohol, polyvinylpyrrolidone, hydroxypropyl guar gum, locust bean gum, amylose, hydroxyethyl amylose, starch and starch derivatives and mixtures thereof. Preferred nonionic polymers include hydroxyethyl cellulose, polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol, polyacrylamide, hydroxypropyl cellulose, ethylhydroxyethyl cellulose, dextran, polypropyleneoxide and hydroxypropyl guar.

Suitable anionic water-soluble polymers include carboxymethyl cellulose, carrageenan, xanthum gum polysty-

rene sulfonate, gum agar, gum ghatti, gum karaya, pectins, alginate salts, as well as poly(acrylic acid) and acrylic or methacrylic acid derivatives such as the alkali metal and ammonium salts of acrylic acid, methacrylic acid. Mixtures of the above anionic water-soluble polymers may also be used.

These polymeric compositions may be homopolymers or they may be copolymers or terpolymers with other copolymerizing monomers known in the art. Examples of copolymerizing monomers known in the art include but are not limited to ethylene, propylene, isobutylene, styrene, polystyrene, alphamethylstyrene, vinyl acetate, vinyl formate, alkyl ethers, acrylonitrile, methacrylonitrile, vinyl chloride, vinylidene chloride, the alkyl acrylates, the alkylmethacrylates, the alkyl fumarates, the alkyl maleates, and other olefinic monomers copolymerizable therewith as long as the resulting polymers are water soluble and phase separate in the compositions of this invention. Copolymers of anionic and nonionic monomers such as acrylic acid and methacrylic acid with acrylamide, methacrylamide, the N-alkyl substituted amides, the N-aminoalkylamides, the corresponding N-alkylaminoalkyl substituted amides, the aminoalkyl acrylates, the aminoalkyl methacrylamides, and the N-alkyl substituted aminoalkyl esters of either acrylic or methacrylic acids.

Preferred anionic polymers include polyacrylic acid; sodium carboxy methyl cellulose; polyacrylates; polymethyl acrylate; polysulphates such as polyvinyl sulfate, polystyrene sulfonate, polyphosphates, sodium dextran sulfate, alginate salts and pectate

When combined with the aqueous surfactant system and phase separation initiator, described below, the water-soluble nonionic or anionic polymer separates to form aqueous droplets suspended in a continuous aqueous phase. The number average particle size of the polymer droplets can be from 0.1 microns to about 10,000 microns, preferably from about 1.0 micron to about 5000 microns, most preferably from about 5 microns to about 1000 microns.

Most preferred for use in the present invention are ethyl hydroxyethyl cellulose, hydroxyethyl cellulose, hydroxypropyl guar and polystyrene sulfonate.

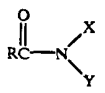
The herein described polymers are preferably present at a concentration level of from above about 0.1% to below 1.0%, more preferably from about 0.15% to about 0.75%, most preferably from about 0.2% to about 0.5%.

Phase Separation Initiators

Another essential component of the present invention is the phase separation initiator. By the term "phase separation initiators", as used herein, means electrolytes, amphiphiles or mixtures thereof capable of inducing phase separation when combined with compositions comprising a surfactant system and a nonionic or anionic water-soluble polymer.

By the term "amphiphile" as used herein, means, generally, substances which contain both hydrophilic and hydrophobic (lipophilic) groups. Amphiphiles preferred for use in the present invention are those which generally do not form micelles or liquid crystal phases and include, but are not limited to: amides of fatty acids; fatty alcohols; fatty esters, glycol mono- and di- esters of fatty acids; glyceryl esters.

Amides, including alkanol amides, are the condensation products of fatty acids with primary and secondary amines or alkanolamines to yield products of the general formula:



wherein RCO is a fatty acid radical and R is C_{8-20}; X is an alkyl, aromatic or alkanol ($CHR'CH_2OH$ wherein R' is H or C_{1-6} alkyl); Y is H, alkyl, alkanol or X. Suitable amides include, but are not limited to, cocamide, lauramide, oleamide and stearamide. Suitable alkanolamides include, but are not limited to, cocamide DEA, cocamide MEA, cocamide MIPA, isostearamide DEA, isostearamide MEA, isostearamide MIPA, lanolinamide DEA, lauramide DEA, lauramide MEA, lauramide MIPA, linoleamide DEA, linoleamide MEA, linoleamide MIPA, myristamide DEA, myristamide MEA, myristamide MIPA, Oleamide DEA, Oleamide MEA, Oleamide MIPA, palmamide DEA, palmamide MEA, palmamide MIPA, palmitamide DEA, palmitamide MEA, palm kernelamide DEA, palm kemelamide MEA, palm kernelamide MIPA, peanutamide MEA, peanutamide MIPA, soyamide DEA, stearamide DEA, stearamide MEA, stearamide MIPA, tallamide DEA, tallowamide DEA, tallowamide MEA, undecylenamide DEA, undecylenamide MEA. The condensation reaction may be carried out with free fatty acids or with all types of esters of the fatty acids, such as fats and oils, and particularly methyl esters. The reaction conditions and the raw material sources determine the blend of materials in the end product and the nature of any impurities.

Fatty alcohols are higher molecular weight, nonvolatile, primary alcohols having the general formula:

$$RCH_2OH$$

wherein R is a C_{8-20} alkyl. They can be produced from natural fats and oils by reduction of the fatty acid COOH— grouping to the hydroxyl function. Alternatively, identical or similarly structured fatty alcohols can be produced according to conventional synthetic methods known in the art. Suitable fatty alcohols include, but are not limited to, behenyl alcohol, C_{9-11} alcohols, C_{12-13} alcohols, C_{12-15} alcohols, C_{12-16} alcohols, C_{14-15} alcohols, caprylic alcohol, cetearyl alcohol, coconut alcohol, decyl alcohol, isocetyl alcohol, isostearyl alcohol, lauryl alcohol, oleyl alcohol, palm kernel alcohol, stearyl alcohol, cetyl alcohol, tallow alcohol, tridecyl alcohol or myristyl alcohol.

Glyceryl esters comprise a subgroup of esters which are primarily fatty acid mono- and di-glycerides or triglycerides modified by reaction with other alcohols and the like. Preferred glyceryl esters are mono and diglycerides. Suitable glyceryl esters and derivatives thereof include, but are not limited to, acetylated hydrogenated tallow glyceride, glyceryl behenate, glyceryl caprate, glyceryl caprylate, glyceryl caprylate/caprate, glyceryl dilaurate, glyceryl dioleate, glyceryl erucate, glyceryl hydroxystearate, glyceryl isostearate, glyceryl lanolate, glyceryl laurate, glyceryl linoleate, glyceryl oleate, glyceryl stearate, glyceryl myristate, glyceryl distearate and mixtures thereof,

Also useful as amphiphiles in the present invention are long chain glycol esters or mixtures thereof. Included are ethylene glycol esters of fatty acids having from about 8 to about 22 carbon atoms. Fatty esters of the formula RCO—OR' also act as suitable amphiphiles in the compositions of the present invention, where one of R and R' is a C_{8-22} alkyl and the other is a C_{1-3} alkyl.

The amphiphiles of the present invention may also encompass a variety of surface active compounds such as

nonionic and cationic surfactants. If incorporated into the compositions of the present invention, these surface active compounds become additional surfactants used as amphiphiles for the purpose of initiating phase separation and are separate and apart from the surfactants of the surfactant system and the alkyl glyceryl sulfonate surfactant of the present invention.

Amphiphiles preferred for use herein include cocamide MEA, cetyl alcohol and stearyl alcohol.

The amphiphiles of the present invention are preferably present in the personal cleansing compositions at levels of from 0 to about 4%. preferably from about 0.5% to about 2%.

Suitable electrolytes include mono-, di- and trivalent inorganic salts as well as organic salts. Surfactant salts themselves are not included in the present electrolyte definition but other salts are. Suitable salts include, but are not limited to, phosphates, sulfates, nitrates, citrates and halides. The counter ions of such salts can be, but are not limited to, sodium, potassium, ammonium, magnesium or other mono-, di and tri valent cation. Electrolytes most preferred for use in the compositions of the present invention include sodium chloride, ammonium chloride, sodium citrate, and magnesium sulfate. It is recognized that these salts may serve as thickening aids or buffering aids in addition to their role as a phase separation initiator. The amount of the electrolyte used will generally depend on the amount of the amphiphile incorporated, but may be used at concentration levels of from about 0.1% to about 4%, preferably from about 0.2% to about 2%.

The amount of phase separation initiator comprising the electrolyte and/or the amphiphile will vary with the type of surfactant and polymer, but is generally present at a level of from about 0.1% to about 5%, preferably from about 0.2% to about 3%.

In view of the essential nature and activity of the phase separation initiators described above, the compositions of the present invention are, preferably, substantially free of materials which would prevent the induction or formation of separate, liquid phases. The term "substantially free", as used here, means that the compositions of the present invention contain no more than about 0.5% of such materials, preferably less than 0.25%, more preferably zero. Such materials typically include ethylene glycol, propylene glycol, ethyl alcohol and the like.

The compositions of the present invention are also preferably substantially free of other ingredients which unduly minimize the formation of separate and distinct liquid phases, especially ingredients which do not provide a significant benefit to the present invention.

Water

The personal cleansing compositions of the present invention comprise from about 50% to about 95%, preferably from about 60% to about 90%, more preferably from about 75% to about 85%, by weight of water.

Optional Ingredients

Silicone Components

The compositions of the present invention may optionally include non-volatile silicone conditioning components. Typically the silicone components are intermixed into aqueous personal cleansing compositions, forming a separate, discontinuous silicone phase. The silicone conditioning component will comprise a silicone fluid conditioning agent such as a silicone fluid and can also comprise other ingredients, such as a silicone resin to enhance silicone fluid deposition efficiency or enhance glossiness of the hair (especially when high refractive index (e.g. above about 1.46) silicone conditioning agents are used (e.g. highly phenylated silicones).

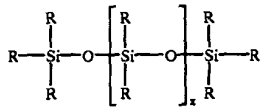
As used herein, "nonvolatile" refers to silicone material with little or no significant vapor pressure under ambient conditions, as is understood by those in the art. Boiling point under one atmosphere (atm) will preferably be at least about 250° C., more preferably at least about 275° C., most preferably at least about 300° C. Vapor pressure is preferably about 0.2mm Hg at 25° C. or less, preferably about 0.1 mm Hg at 25° C. or less.

The silicone conditioning agent phase may comprise volatile silicone, nonvolatile silicone, or mixtures thereof. Typically, if volatile silicones are present, it will be incidental to their use as a solvent or carrier for commercially available forms of nonvolatile silicone materials ingredients, such as silicone gums and resins.

The silicone conditioning agents for use in the compositions of the present invention preferably have a viscosity of from about 20 to about 2,000,000 centistokes, more preferably from about 1,000 to about 1,800,000 centistokes, even more preferably from about 10,000 to about 1,500,000 centistokes, most preferably from about 30,000 to about 1,000,000 centistokes, at 25° C. The viscosity can be measured by means of a glass capillary viscometer as set forth in Dow Corning Corporate Test Method CTM0004, Jul. 20, 1970.

Optional silicone fluid for use in the present compositions include silicone oils which are flowable silicone materials with a viscosity of less than 1,000,000 centistokes, preferably between about 5 and 1,000,000 centistokes, more preferably between about 10 and about 600,000 centistokes, more preferably between about 10 and about 500,000 centistokes, most preferably between 10 and 300,000 centistokes at 25° C. Suitable silicone oils include polyalkyl siloxanes, polyaryl siloxanes, polyalkylaryl siloxanes, polyether siloxane copolymers, and mixtures thereof. Other insoluble, nonvolatile silicone fluids having conditioning properties can also be used.

Optional Silicone oils for use in the composition include polyalkyl or polyaryl siloxanes which conform to following formula:



where R is aliphatic, preferably alkyl or alkenyl, or aryl, R can be substituted or unsubstituted, and x is an integer from 1 to about 8,000. Suitable unsubstituted R groups include alkoxy, aryloxy, alkaryl, arylalkyl, arylalkenyl, alkamino, and ether-substituted, hydroxyl-substituted, and halogen-substituted aliphatic and aryl groups. Suitable R groups also include cationic amines and quaternary ammonium groups.

The aliphatic or aryl groups substituted on the siloxane chain may have any structure as long as the resulting silicones remain fluid at room temperature, are hydrophobic, are neither irritating, toxic nor otherwise harmful when applied to the hair or skin, are compatible with the other components of the herein described personal cleansing compositions, are chemically stable under normal use and storage conditions, are insoluble in the compositions of the present invention, and are capable of being deposited on and, of conditioning, the hair and skin.

The two R groups on the silicon atom of each monomeric silicone unit may represent the same group or different groups. Preferably, the two R groups represent the same group.

Preferred alkyl and alkenyl substituents are C_1–C_5 alkyls and alkenyls, more preferably from C_1–C_4, most preferably from C_1–C_2. The aliphatic portions of other alkyl-, alkenyl-, or alkynyl-containing groups (such as alkoxy, alkaryl, and alkamino) can be straight or branched chains and preferably have from one to five carbon atoms, more preferably from one to four carbon atoms, even more preferably from one to three carbon atoms, most preferably from one to two carbon atoms. As discussed above, the R substituents hereof can also contain amino functionalities, e.g. alkamino groups, which can be primary, secondary or tertiary amines or quaternary ammonium. These include mono-, di- and trialkylamino and alkoxyamino groups wherein the aliphatic portion chain length is preferably as described above. The R substituents can also be substituted with other groups, such as halogens (e.g. chloride, fluoride, and bromide), halogenated aliphatic or aryl groups, and hydroxy (e.g. hydroxy substituted aliphatic groups). Suitable halogenated R groups could include, for example, tri-halogenated (preferably fluoro) alkyl groups such as $—R^1—C(F)_3$, wherein R^1 is C_1–C_3 alkyl. Examples of such polysiloxanes include polymethyl-3,3,3 trifluoropropylsiloxane.

Suitable R groups include methyl, ethyl, propyl, phenyl, methylphenyl and phenylmethyl. The preferred silicones are polydimethyl siloxane, polydiethylsiloxane, and polymethylphenylsiloxane. Polydimethylsiloxane is especially preferred. Other suitable R groups include methyl, methoxy, ethoxy, propoxy, and aryloxy. The three R groups on the end caps of the silicone may also represent the same or different groups.

The nonvolatile polyalkylsiloxane fluids that may be used include, for example, polydimethylsiloxanes. These siloxanes are available, for example, from the General Electric Company in their Viscasil R and SF 96 series, and from Dow Corning in their Dow Corning 200 series.

The polyalkylaryl siloxane fluids that may be used, also include, for example, polymethylphenylsiloxanes. These siloxanes are available, for example, from the General Electric Company as SF 1075 methyl phenyl fluid or from Dow Corning as 556 Cosmetic Grade Fluid.

The polyether siloxane copolymers that may be used include, for example, a polypropylene oxide modified polydimethylsiloxane (e.g., Dow Corning DC-1248) although ethylene oxide or mixtures of ethylene oxide and propylene oxide may also be used. The ethylene oxide and polypropylene oxide level must be sufficiently low to prevent solubility in water and the composition hereof.

Other suitable silicone fluids for use in the silicone conditioning agents are insoluble silicone gums. These gums are polyorganosiloxane materials having a viscosity at 25° C. of greater than or equal to 1,000,000 centistokes. Silicone gums are described in U.S. Pat. No. 4,152,416; Noll and Walter, *Chemistry and Technology of Silicones,* New York: Academic Press 1968; and in General Electric Silicone Rubber Product Data Sheets SE 30, SE 33, SE 54 and SE 76, all of which are incorporated herein by reference. The silicone gums will typically have a mass molecular weight in excess of about 200,000, generally between about 200,000 and about 1,000,000, specific examples of which include polydimethylsiloxane, (polydimethylsiloxane) (methylvinylsiloxane) copolymer, poly(dimethylsiloxane) (diphenyl siloxane)(methylvinylsiloxane) copolymer and mixtures thereof.

The silicone conditioning agent can also comprise a mixture of polydimethylsiloxane gum (viscosity greater than about 1,000,000 centistokes) and polydimethylsiloxane oil (viscosity from about 10 to about 100,000 centistokes), wherein the ratio of gum to fluid is from about 30:70 to about 70:30, preferably from about 40:60 to about 60:40.

References disclosing examples of some suitable silicone fluids for use in the personal cleansing compositions include U.S. Pat. No. 2,826,551, U.S. Pat. No. 3,964,500, U.S. Pat. No. 4,364,837, British Patent 849,433, and *Silicon Compounds,* Petrarch Systems, Inc. (1984), all of which are incorporated herein by reference.

Silicone resins can be included in the silicone conditioning agent. These resins are highly crosslinked polymeric siloxane systems. The crosslinking is introduced through the incorporation of trifunctional and tetrafunctional silanes with monofunctional or difunctional, or both, silanes during manufacture of the silicone resin. As is well understood in the art, the degree of crosslinking that is required in order to result in a silicone resin will vary according to the specific silane units incorporated into the silicone resin. In general, silicone materials which have a sufficient level of trifunctional and tetrafunctional siloxane monomer units (and hence, a sufficient level of crosslinking) such that they dry down to a rigid, or hard, film are considered to be silicone resins. The ratio of oxygen atoms to silicon atoms is indicative of the level of crosslinking in a particular silicone material. Silicone materials which have at least about 1.1 oxygen atoms per silicon atom will generally be silicone resins herein. Preferably, the ratio of oxygen:silicon atoms is at least about 1.2:1.0. Silanes used in the manufacture of silicone resins include monomethyl-, dimethyl-, trimethyl-, monophenyl-, diphenyl-, methylphenyl-, monovinyl-, and methylvinyl-chlorosilanes, and tetrachlorosilane, with the methyl-substituted silanes being most commonly utilized. Preferred resins are offered by General Electric as GE SS4230 and SS4267. Commercially available silicone resins will generally be supplied in a dissolved form in a low viscosity volatile or nonvolatile silicone fluid. The silicone resins for use herein should be supplied and incorporated into the present compositions in such dissolved form, as will be readily apparent to those skilled in the art.

Background material on silicones including sections discussing silicone fluids, gums, and resins, as well as manufacture of silicones, can be found in Encyclopedia of Polymer Science and Engineering, Volume 15, Second Edition, pp. 204–308, John Wiley & Sons, Inc., 1989, incorporated herein by reference.

Silicone materials and silicone resins in particular, can conveniently be identified according to a shorthand nomenclature system well known to those skilled in the art as "MDTQ" nomenclature. Under this system, the silicone is described according to presence of various siloxane monomer units which make up the silicone. Briefly, the symbol M denotes the monofunctional unit $(CH_3)_3SiO_{0.5}$; D denotes the difunctional unit $(CH_3)_2SiO$; T denotes the trifunctional unit $(CH_3)SiO_{1.5}$; and Q denotes the quadri- or tetrafunctional unit SiO_2. Primes of the unit symbols, e.g., M', D', T', and Q' denote substituents other than methyl, and must be specifically defined for each occurrence. Typical alternate substituents include groups such as vinyl, phenyls, amines, hydroxyls, etc. The molar ratios of the various units, either in terms of subscripts to the symbols indicating the total number of each type of unit in the silicone (or an average thereof) or as specifically indicated ratios in combination with molecular weight complete the description of the silicone material under the MDTQ system. Higher relative

molar amounts of T, Q, T' and/or Q' to D, D', M and/or M' in a silicone resin is indicative of higher levels of crosslinking. As discussed before, however, the overall level of crosslinking can also be indicated by the oxygen to silicon ratio.

The silicone resins for use herein which are preferred are MQ, MT, MTQ, MDT and MDTQ resins. Thus, the preferred silicone substituent is methyl. Especially preferred are MQ resins wherein the M:Q ratio is from about 0.5:1.0 to about 1.5:1.0 and the average molecular weight of the resin is from about 1000 to about 10,000.

The weight ratio of the nonvolatile silicone fluid, having refractive index below 1.46, to the silicone resin component, when used, is preferably from about 4:1 to about 400:1, preferably this ratio is from about 9:1 to about 200:1, more preferably from about 19:1 to about 100:1, particularly when the silicone fluid component is a polydimethylsiloxane fluid or a mixture of polydimethylsiloxane fluid and polydimethylsiloxane gum as described above. Insofar as the silicone resin forms a part of the same phase in the compositions hereof as the silicone fluid, i.e. the conditioning active, the sum of the fluid and resin should be included in determining the level of silicone conditioning agent in the composition.

The number average particle size of the optional silicone component can vary widely depending on the formulation and/or the desired characteristics. Number average particle sizes preferred for use in the present invention range from about 10 nanometers to about 100 microns, more preferably from about 30 nanometers to about 20 microns.

Other Optional Components

The personal cleansing compositions of the present invention may further comprise one or more optional components known for use in shampoo, conditioning and other personal cleansing compositions, provided that the optional components are physically and chemically compatible with the essential component described herein, or do not otherwise unduly impair product stability, aesthetics or performance. Concentrations of such optional components typically range from about 0.001% to about 30% by weight of the personal cleansing compositions.

Optional components include anti static agents, cationic conditioning polymers such as polyquaterinum-10, dyes, organic solvents or diluents, emollient oils (such as polyisobutylene, mineral oil, petrolatum and isocetyl stearyl stearate), pearlescent aids, foam boosters, pediculocides, pH adjusting agents, perfumes, preservatives, proteins, antioxidants; chelators and sequestrants; and aesthetic components such as fragrances, colorings, essential oils, skin sensates, astringents, skin soothing agents, skin healing agents and the like, nonlimiting examples of these aesthetic components include panthenol and derivatives (e.g. ethyl panthenol), pantothenic acid and its derivatives, clove oil, menthol, camphor, eucalyptus oil, eugenol, menthyl lactate, witch hazel distillate, allantoin, bisabalol, dipotassium glycyrrhizinate and the like, suspending agents, styling polymers, sunscreens, thickeners, vitamins and derivatives thereof (e.g., ascorbic acid, vitamin E, tocopheryl acetate, retinoic acid, retinol, retinoids, and the like), and viscosity adjusting agents. This list of optional components is not meant to be exclusive, and other optional components can be used.

Method of Manufacture

The compositions of the present invention, in general, can be made by mixing together at elevated temperature, e.g., about 72° C. water and surfactants along with any solids (e.g., amphiphiles) that need to be melted, to speed mixing into the personal cleansing composition. Additional ingredients including the electrolytes can be added either to this hot premix or after cooling the premix. The nonionic or anionic polymers can be added as a water solution after cooling the premix. The ingredients are mixed thoroughly at the elevated temperature and then pumped through a high shear mill and then through a heat exchanger to cool them to ambient temperature. The silicone may be emulsified at room temperature in concentrated surfactant and then added to the cooled product. Alternately, for example, the silicone conditioning agent can be mixed with anionic surfactant and fatty alcohol, such as cetyl and stearyl alcohols, at elevated temperature, to form a premix containing dispersed silicone. The premix can then be added to and mixed with the remaining materials of the personal cleansing composition, pumped through a high shear mill, and cooled.

Method of Use

The personal cleansing compositions of the present invention are used in a conventional manner for cleansing and conditioning hair or skin. An effective amount of the composition for cleansing and conditioning the hair or skin is applied to the hair or skin, that has preferably been wetted with water, and then rinsed off. Such effective amounts generally range from about 1 g to about 50 g, preferably from about 1 g to about 20 g. Application to the hair typically includes working the composition through the hair such that most or all of the hair is contacted with the composition.

This method for cleansing and conditioning the hair and skin comprises the steps of: a) wetting the hair and/or skin with water, b) applying an effective amount of the personal cleansing composition to the hair and/or skin, and c) rinsing the composition from the hair and/or skin using water. These steps can be repeated as many times as desired to achieve the desired cleansing and conditioning benefit.

Examples

The personal cleansing compositions illustrated in Examples I–X illustrate specific embodiments of the personal cleansing compositions of the present invention, but are not intended to be limiting thereof. Other modifications can be undertaken by the skilled artisan without departing from the spirit and scope of this invention. These exemplified embodiments of the personal cleansing compositions of the present invention provide cleansing of hair and/or skin and improved conditioning.

All exemplified compositions can be prepared by conventional formulation and mixing techniques. Component amounts are listed as weight percents and exclude minor materials such as diluents, filler, and so forth. The listed formulations, therefore, comprise the listed components and any minor materials associated with such components.

Ingredients	I	II	III	IV	V
Ammonium Laureth Sulfate	5.00	—	—	—	—
Ammonium Lauryl Sulfate	5.00	7.50	7.50	7.50	7.50
Sodium alkyl glycerol sulfonate	2.50	2.50	2.50	2.50	2.50
Cocoamidopropyl Betaine	—	—	—	—	—
Glycol Distearate	2.00	1.50	2.00	2.00	2.00
Cocomonoethanol amide	0.60	0.85	0.85	0.85	0.85
Cetyl Alcohol	0.42	0.42	0.42	0.42	0.42
Stearyl Alcohol	0.18	0.18	0.18	0.18	0.18
EDTA (ethylenediamine tetra acetic acid)	0.10	0.10	0.10	0.10	0.10

-continued

Monosodium phosphate	0.10	0.10	0.10	0.10	0.10
Disodium phosphate	0.20	0.20	0.20	0.20	0.20
Sodium Benzoate	0.25	0.25	0.25	0.25	0.25
Hydroxyethylcellulose[1]	0.10	0.25	—	—	—
Hydroxypropyl Guar[2]	—	—	0.25	—	—
Hydroxyethylethylcellulose[3]	—	—		0.25	—
Polystyrene Sulfonate	—	—		—	0.25
Tricetyl methylammonium chloride	0.58	—	—	—	—
Perfume	0.60	0.60	0.60	0.60	0.60
Dimethicone	1.00	1.50	1.50	1.50	1.50
Glydant	0.20	0.20	0.20	0.20	0.20
NaCl	0.30	0.30	1.00	1.00	1.00
Water and minors	q.s. to 100%				

Ingredients	VI	VII	VIII	IX	X
Ammonium Laureth Sulfate	—	9.00	14.00	6.60	8.00
Ammonium Lauryl Sulfate	6.00	—	—	6.60	—
Sodium alkyl glycerol sulfonate	1.00	2.50	—	—	—
Cocoamidopropyl Betaine	—	2.50	2.70	—	—
Glycol Distearate	1.50	1.50	1.50	1.50	2.00
Cocomonoethanol amide	0.85	0.85	0.85	0.85	—
Cetyl Alcohol	0.42	0.42	0.42	0.42	0.40
Stearyl Alcohol	0.18	0.18	0.18	0.18	0.18
EDTA (ethylenediamine tetra acetic acid)	0.10	0.10	0.10	0.10	0.10
Monosodium phosphate	0.10	0.10	0.10	0.10	0.10
Disodium phosphate	0.20	0.20	0.20	0.20	0.20
Sodium Benzoate	0.25	0.25	0.25	0.25	0.25
Hydroxyethylcellulose[1]	0.25	0.25	0.10	0.75	0.25
Hydroxypropyl Guar[2]	—	—	—	—	—
Hydroxyethylethylcellulose[3]	—	—	—	—	—
Polystyrene Sulfonate	—	—	—	—	—
Tricetyl methylammonium chloride	—	—	—	—	—
Perfume	0.60	0.60	0.60	0.60	0.60
Dimethicone	1.50	1.50	1.50	—	—
Glydant	0.20	0.20	0.20	0.20	0.20
Sodium Lauroamphoacetate	—	—	—	—	3.60
Polyquaternium-10	—	—	—	—	0.20
NaCl	0.30	0.30	0.30	0.30	—
Water and minors	q.s. to 100%				

[1]Natrosol 250 HHR from Aqualon
[2]Jaguar HP 60 from Rhone-Poulenc
[3]Bermocoll E411 FQ from Akzo Nobel

What is claimed is:

1. A personal cleansing composition in the form of a stable aqueous emulsion, comprising:

a.) from about 4% to about 50%, by weight, of a surfactant system comprising at least one surfactant selected from the group consisting of anionic, cationic, nonionic, amphoteric, and zwitterionic surfactants and mixtures thereof,

b.) from above about 0.1% to less than 1.0%, by weight, of a water soluble polymer selected from the group consisting of hydroxyethyl cellulose, polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol, polyacrylamide, hydroxypropyl cellulose, ethylhydroxyethyl cellulose, dextran, polypropyleneoxide, polystyrene, sodium carboxy methyl cellulose, polysulphates, polyphosphates, sodium dextran sulfate, alginate, pectate, and mixtures thereof;

c.) from about 0.1% to about 5%, by weight, of a phase separation initiator selected from the group consisting of electrolytes, amphiphiles and mixtures thereof; and

d.) from about 50% to about 95%, by weight, of water

wherein said polymer forms visually distinct aqueous droplets in the aqueous surfactant system.

2. A personal cleansing composition according to claim 1, wherein the number average particle size of the polymer droplets is greater than about 0.1 microns.

3. A personal cleansing composition according to claim 1, wherein the surfactant system is present at a concentration of from about 4% to about 30%.

4. A personal cleansing composition according to claim 3, wherein the surfactant system is present at a concentration of from about 5% to about 20%.

5. A personal cleansing composition according to claim 1, wherein the nonionic or anionic polymer is selected from the group consisting of hydroxyethyl cellulose, polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol, polyacrylamide, hydroxypropyl cellulose, ethylhydroxyethyl cellulose, dextran, polypropyleneoxide, hydroxypropyl guar, guar gums, polyacrylic acid, polystyrene, sodium carboxy methyl cellulose, polycarboxylates, polysulphates, polyphosphates, sodium dextran sulfate, alginate, pectate, derivatives thereof and mixtures thereof.

6. A personal cleansing composition according to claim 5, wherein the nonionic and anionic polymer is selected from the group consisting of hydroxyethyl cellulose, ethylhydroxyethyl cellulose, hydroxypropyl guar, polystyrene and mixtures thereof.

7. A personal cleansing composition according to claim 1, wherein the surfactant system comprises surfactants selected from the group consisting of ammonium lauryl sulfate, ammonium laureth sulphate, cocamidopropyl betaine, sodium lauroamphoacetate, alkyl glyceryl ether sulfonate derivatives thereof and mixtures thereof.

8. A personal cleansing composition according to claim 1, wherein the amphiphile is selected from the group consisting of cocamide MEA, cetyl alcohol, stearyl alcohol, derivatives thereof and mixtures thereof.

9. A personal cleansing composition according to claim 1, wherein the electrolyte is selected from the group of consisting of anions selected from the group consisting of phosphates, sulfates, nitrates, citrates, halides; cations selected from the group consisting of sodium, potassium, ammonium, magnesium; and mixtures thereof.

10. A personal cleansing composition according to claim 1, further comprising a silicone conditioning component.

11. A silicone conditioning component according to claim 10, wherein the number average particle size of the silicone is from about 10 nanometers to about 100 microns.

12. A personal cleansing composition according to claim 1, in the form of a shampoo, foam, mousse, gel, lotion, spray, liquid soap, shower gel or lotion.

13. A personal cleansing composition in the form of a stable aqueous emulsion, comprising:

a.) from about 4% to about 50%, by weight, of a surfactant system comprising at least one surfactant selected from the group consisting of anionic, cationic, nonionic, amphoteric, and zwitterionic surfactants and mixtures thereof;

b.) from above about 0.1% to less than 1.0%, by weight, of a water soluble polymer selected from the group consisting of hydroxyethyl cellulose polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol, polyacrylamide, hydropropyl cellulose, ethylhydroxyethyl cellulose, dextran, polypropyleneoxide, polystyrene, sodium carboxy methyl cellulose, polysulphates, polyphosphates, sodium dextran sulfate, alginate, pectate, and mixtures thereof;

c.) from about 0.1% to about 4%, by weight, of an electrolyte;

d.) from about 0% to about 4%, by weight, of an amphiphile; and

e.) from about 50% to about 95%, by weight, of water

wherein said polymer forms visually distinct aqueous droplets in the aqueous surfactant system.

14. A personal cleansing composition according to claim 13, wherein the nonionic and anionic polymer is selected

from the group consisting of hydroxyethyl cellulose, ethylhydroxyethyl cellulose, hydroxypropyl guar, polystyrene and mixtures thereof.

15. A personal cleansing composition according to claim 13, wherein the surfactant system comprises surfactants selected from the group consisting of ammonium lauryl sulfate, ammonium laureth sulphate, cocamidopropyl betaine, sodium lauroamphoacetate, alkyl glyceryl ether sulfonate derivatives thereof and mixtures thereof.

16. A personal cleansing composition according to claim 13, wherein the amphiphile is selected from the group consisting of cocamide MEA, cetyl alcohol, stearyl alcohol, and mixtures thereof.

17. A personal cleansing composition according to claim 13, wherein the electrolyte is selected from the group of consisting of anions selected from the group consisting of phosphates, sulfates, nitrates, citrates, halides; cations selected from the group consisting of sodium, potassium, ammonium, magnesium; and mixtures thereof.

18. A method of treating hair by administering a safe and effective amount of the compositions according to claim 1.

19. A method of treating skin by administering a safe and effective amount of the compositions according to claim 1.

20. A method of cleaning hair and skin by administering a safe and effective amount of the compositions according to claim 1.

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